THESIS

QUANTITATIVE ANALYSIS OF CERTAIN ANTIANGINAL DRUGS IN PHARMACEUTICAL FORMULATIONS

ABSTRACT

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

CHEMISTRY

BY

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ALIGARH MUSLIM UNIVERSITY
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The thesis entitled ***"Quantitative Analysis of Certain Anatianginal Drugs in Pharmaceutical Formulations"*** is comprised of five chapters. The first chapter describes a general literature survey of the subject matter. The very relevant matters include, a brief discussion on analytical chemistry, its relation with our daily life from the old age, role played in the field of pharmaceutical and biomedical analysis; various types of analytical techniques which are frequently used in the field under discussion; the validation of the developed method and the statistical treatments adopted during the data analysis to ensure and enforce the validity of the method.

It is well known that the human nature is the most cautious in every era of the development. This lead to the birth of analytical chemistry as it deals with the study of the intrinsic properties of the materials to discover their valuable applicability or to warn the concerned one with the possibility of harm from them. Unlike other areas of studies in science, there are different properties, which heavily influence the results of the analytical methodology at each and every step; thus having direct impact on the results. These important properties have been discussed. With the passing time, the demands of the biochemical sciences and the advancement of the physical sciences opened the door of sophisticated instrumentation in the field of analytical chemistry. The different types of well-established analytical techniques have been discussed.

Before or during the development of a method, it is necessary to be very careful of certain important things. These concepts have been discussed briefly. Once the analytical method is advent, it is necessary to decide its suitability for the intended purpose. This is known as the method validation. Brief discussions of the validation, its components and the different international organisation involved in it, have been presented. When deciding for the

validation of the method, the role of statistical analysis can not be ignored. It is the only way to get the most conclusive results from the mathematical data obtained during the analysis. It also helps to decide with the progress of the work at each step. A detailed discussion of the statistical analysis has been given.

As clear from the title of the thesis, it is based on the assay of certain specific pharmaceuticals i.e. antianginal drugs. A brief literature and classification of the concerned pharmaceuticals have also been presented.

The second chapter describes the spectrophotometric method for the determination of nifedipine in drug formulations. The method depends on the reduction of nitro group to hydroxylamino group, which is then reacted with N–methyl–1,4–benzoquinoneimine to form coloured product (*525 nm*). The experimental conditions were optimised. Under the optimum experimental conditions Beer's law was obeyed over the concentration range of *5 – 175 μg* mL^{-1}. The molar absorptivity, detection limit, recovery and RSD were found to be 1.9×10^3 *L* mol^{-1} cm^{-1}, *1.1 μg* mL^{-1}, *99.7 – 100.5%* and *0.3 – 0.8%*, respectively. The proposed method was compared favourably with the official B.P. method.

The third chapter deals with a simple, rapid and economical spectrophotometric method for the assay of verapamil hydrochloride. Aqueous solution of verapamil is oxidised by the aqueous solution of chloramine–T in hydrochloric acid medium resulting in the formation of yellow coloured chromophore which absorbed maximally at *425 nm*. The colour becomes constant within 13 minutes and remains so upto 20 minutes. The Beer's range was found to be *0 – 340 μg* mL^{-1}. Rigorous statistical analysis of the calibration data was done to check and validate the strong correlation of the slope and intercept obtained for the regression equation. A plot of joint confidence by the method of Mandel and Linnig was drawn which results in an

ellipse having its centre at the point of best fit. The obtained experimental data show excellent linearity *(r = 0.9999).* The application of this method on the dosage forms yields accurate and precise results. No interference from the commonly added adjuvants like carbohydrates was encountered as chloramine–T oxidises them only in the alkaline medium. This is a significant advantage.

Two spectrophotometric methods for the assay of amlodipine besylate have been included in the fourth chapter. The first method is based on the charge transfer complexation reaction of amlodipine base molecule acting as the donor with that of the 2,3–dichloro 5,6–dicyano 1,4–benzoquinone (DDQ) as acceptor in acetonitrile medium. Amlodipine base was previously obtained in bulk form in chloroform by the treatment of amlodipine besylate with aqueous sodium carbonate solution. The red coloured DDQ radical anion formed absorbed maximally at *435, 550* and *580 nm.* In this method the determination was done at *580 nm.* In the second method ascorbic acid was used as the reagent to develop a purple coloured complex (λ_{max} *530 nm*) with the aliphatic $-NH_2$ group of amlodipine molecule. In 1964, J. Bartos introduced ascorbic acid as a reagent for the detection and determination of aliphatic amino group. Since then the mechanism of the reaction has not been exactly elucidated. In this section the interest was also shown to propose the relevant reaction mechanism. For this purpose FTIR studies of the analyte and the reaction product were done. A tentative reaction mechanism has been given. The Beer's law was obeyed in the concentration range *1 – 125* and *10 – 140 μg* mL^{-1} for DDQ and ascorbic acid methods, respectively. The molar absorptivity, slope, intercept and its confidence interval, correlation coefficient, linearity, standard analytical error, and confidence limit were calculated. The variances were found to be very low (*4.44* $x10^{-6}$ and *4.50* $x10^{-5}$ for DDQ and ascorbic acid methods, respectively). The

results obtained by applying the proposed methods on the locally available formulations were compared with that of the reference method using Student's *t*- and variance ratio *F*- tests. The joint confidence test was also applied to justify the existing relation of the slope and the intercept.

In the last chapter of the thesis, the kinetic spectrophotometric determination of diltiazem hydrochloride has been included. To the best of our knowledge this is the first attempt to develop a kinetic spectrophotometric method for the determination of diltiazem hydrochloride in pharmaceutical formulations. The method is based on the oxidation of diltiazem hydrochloride by potassium permanganate in alkaline aqueous medium. A tentative reaction mechanism has been given. Four different methods of possible calibration curves have been proposed: The plots of **(i)** log intercept vs. log of molar concentration of diltiazem hydrochloride *(log C);* **(ii)** log initial rate of formation of MnO_4^{-2} vs. *log C;* **(iii)** absorbance *(610 nm)* measured at a fixed time of 14 minutes vs. concentration; and **(iv)** log rate of degradation of potassium permanganate *(530 nm)* vs. *log C,* showed a linear dynamic range of $1-7$, $1-5$, $1-7$ and $4-12\ \mu g\ mL^{-1}$, respectively. Different types of tests for the outliers in the data like Dixon's test and Grubb's test, test for normal distribution (David test) and test for the homoscedasticity of variances (Cochran test) were also performed. Detailed accuracy and precision studies have been done on both the pure as well as the dosage forms. Analysis of variance for the lack of fit test and for repeatability and intermediate precision were performed.

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Dedicated

to

My Teachers

THESIS

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Certificate

This is to certify that the thesis entitled **"Quantitative Analysis of Certain Antianginal Drugs in Pharmaceutical Formulations"** is the original work of Mr. **Md. Nasrul Hoda**, carried out under my supervision and is suitable for submission for the award of the degree of Doctor of Philosophy in Chemistry.

Nafisur Rahman

(Nafisur Rahman)

Acknowledgement

I bring all the praises and commendation to the Almighty for providing me with strength during the work of my research which helped me to overcome the troubles in the toilsome journey.

It will be hard to find out the words to thank my sage supervisor, Dr. Nafisur Rahman, for his excellent guidance, constant support and trust throughout the course of my research work.

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I express my deep sense to my parents whose blessings have always served as a sheet anchor against the mundane odds and inspired me to complete my mission. I am equally be holding to my elder sisters, Advocate Meenu and Hina.

It will not be justified, if I don't acknowledge the contributions of my wife who is suffering my affection to the science but always stands with me in adversity and prosperity with continuous strive and endeavour.

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(Md. Nasrul Hoda)

Contents

LIST OF PUBLICATIONS

[1]. Spectrophotometric method for the determination of nifedipine with 4–(methylamino)phenol and potassium dichromate; ***Il Farmaco (Elsevier, France)***, 57 (2002) 435–441.

[2]. Spectrophotometric determination of verapamil hydrochloride in drug formulations with chloramine–T as oxidant; ***Anal. Bioanal. Chem. (Springer–Verlag, Germany)***, 374 (2002) 484–489.

[3]. Validated spectrophotometric methods for the determination of amlodipine besylate in drug formulations using 2,3–dichloro 5,6–dicyano 1,4–benzoquinone and ascorbic acid; ***J. Pharm. Biomed. Anal. (Elsevier, Ireland)***, (In press).

[4]. Kinetic spectrophotometric methods for the determination of diltiazem hydrochloride in drug formulations; ***Anal. Sci. (Japan)***, (Communicated).

LIST OF FIGURES

LIST OF TABLES

Chapter - 1

General Introduction

In the context of the present knowledge, the less predictive experiments, observations and interpretations of the ancient philosophers and scholars must be regarded as purely heuristic which were based on the superstitions and misunderstandings of the natural phenomena. Nevertheless, the account of those deeds of antiquity, the middle ages and even the first 350 years of the "**modern age**" make fascinating anecdotal reading. But even today it is a customary in good sense and justified too, to credit them who have, in fact, smoothen the pathway for the recent sophisticated and conclusive research works.

It was **Aristotle** who had anticipated the nature of the hormones and claimed that the humours were secreted at certain cites to act elsewhere in the body and thus gave birth to the **Chemical Science**, and liberated it from the preconceived ideas of mysterious forces. During the period regarded as the "**Quintessence to Chemical**" (Paracelsus, 16th century–Ehrlich, 19th century) [1], people had undergone major gradual change in the use of natural products in their entire states (mainly having medicinal importance) to either the purified extracts of the material of interest from those or to synthetic chemically–produced materials.

Louis Pasteur (1822–1895) had given a great breakthrough to the **chemotherapy** for the treatment of infectious diseases caused by the pathogenic parasites. This lead to a wave in antiprotozoal chemotherapy system around 1890, which forced to develop some hypnotic, antimalarial and anti-inflammatory drugs, and adrenergic and cholinergic hormones, followed by the discovery of antibacterial and antibiotics, pharmacodynamics of analgesics, antihistaminics, vitamins and few new hormones. The post World War–II period had given major breakthrough to the drug development and a number of antituberculous agents, the steroid hormones and contraceptives, antipsychotic, anxiolytic and

antidepressant psycho-pharmacological drugs were discovered. And finally with the enforcement of **Pure Food and Drug Act** by the Food and Drug Administration of the United States, the therapeutic research, drug development, introduction and pharmaceutical manufacturing entered their "**Golden Age**" (1940–1960).

With the cautious nature of human being and growing awareness especially for the health hazard reasons, the concept of **quality** emerged which lead to the interest in the work of **analysis**. With no name designated and no specificity given in the old age, **analytical chemistry** is the important part of the good quality life in every period of the development. The very basic aspect of analytical chemistry defines it as "*the relationship between the so-called intrinsic chemical information of the objects and systems* [2] *and the information (results, reports) provided by the analytical systems (laboratories, on-site analysers etc.)*". A book written by **Thomas De Quincey** [3] can well document the old age relation of analytical chemistry with our daily life. In his book "**Confessions of an English Opium Eater**", the man of letters penned about his surgeon friend who was suffering from a lingering and fatal malady. The surgeon was on the horns of a dilemma as he had a family to support and thus eager to continue in work. Because of his professional knowledge, he saw the necessity of reducing his daily dose of opium that he took to alleviate his pains to a minimum. I now quote the words of De Quincey in this context: "*But to do this he must first obtain the means of measuring the quantities of opium; not the "**apparent quantities**" as determined by weighing, but the "**virtual quantities**" after allowing for the alloy of varying amounts of impurities*". With the skills of the time (about 1820) De Quincey's surgeon friend was unable for such an evaluation. He did, however, achieve a uniform method of extracting the opium so as to give a fairly constant potency. G.D. Christian [4] has traced, in a report, the evolution of

analytical chemistry into the 20th century and the revolution in quantitative analysis using modern analytical instruments.

The chemical analysis is divided into qualitative and quantitative types of analyses. The qualitative analysis deals with the detection of constituents or components present in the analyte under investigation. The quantitative analysis determines the proportions in which the constituents or the amount of constituents are present in the matter of interest.

Undoubtedly, as far as the quality in the analytical results is concerned, the top aim of the analytical chemists is to minimise the differences between the ideal, true information and that produced in the routine work. This primary goal of analytical chemist relies heavily on two capital analytical properties: the accuracy and the representativeness. In the search of excellency in these aspects, the analytical chemists worldwide are continuously making their best efforts for developing the sophisticated (accurate and precise) and sensitive techniques. Here the technical aspect is not including the instrumentation only, but also the various types of other trial like the suitable modifications in the methods of estimation, certain changes in the traditional ways of conducting the chemical reactions and sometimes developing new theoretical concepts for the old/previously existing instruments/methods.

Not only the different theoretical aspects or the advance instrumentation (whether it is old or new) are affecting the quality in the analytical results but also it is seriously influenced by the different analytical properties. These analytical properties may be classified as the ***capital*** (accuracy and representativeness), ***basic*** (sensitivity, selectivity, precision and sampling) and ***accessory*** (expeditiousness, cost-effectiveness and personal-related considerations like personal safety and comfort). The capital analytical properties are associated with the

consistency in the results and strongly rely on the basic properties which are responsible for the quality in the output of both inter as well as intra-laboratory analytical processes. Accessory analytical properties, though seems to be less significant, but often have major practical implications and sometimes affect the output heavily. Thus the basic and accessory properties are related to the analytical methodology. The hierarchy of these important components has been presented (Fig. 1.1) while their interdependence is shown in Fig. 1.2.

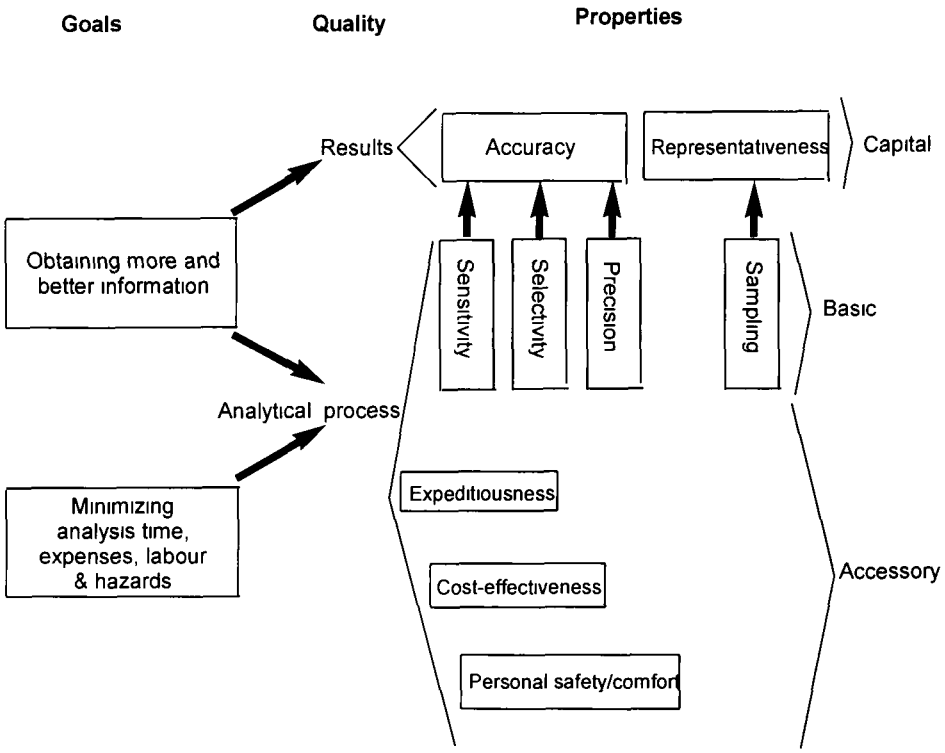


Figure 1.1. Goals of analytical chemistry and their relationships to the analytical quality and analytical properties.

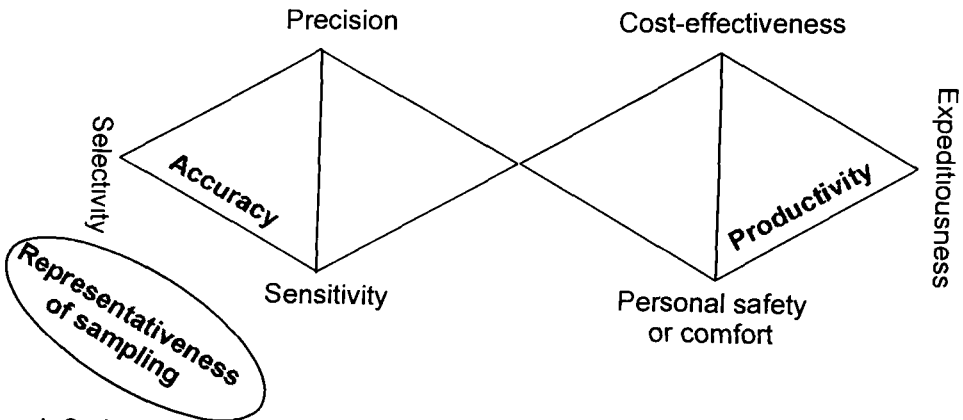


Figure 1.2. Analytical tetrahedra showing interdependence of the analytical properties.

An investigation of Fig. 1.1 shows that these are the basic properties, which are mainly deciding with the top goal of the analytical methodology, i.e. accuracy and representativeness. In fact these basic properties are themselves interdependent and affect each other strongly. Due to this strong relation, the excellency or even a simple enhancement in any of the basic property can only be achieved at the expense of another one. The present generic objective of the analytical chemistry is to get the high quality analytical information by expending minimum material, less time with least number of the man-power and minimum risks at the lowest cost of expense.

As far as the pharmaceutical analysis is concerned, it always needs for the sensitive and sophisticated techniques. The most frequently used analytical techniques in pharmaceutical analysis are titrimetry, chromatography, electrochemical analysis and spectroscopy. Sometimes these techniques are combined with other supporting/subsidiary instruments like flow injection analysis system or kinetic type of analysis; thus making them more sensitive, selective and fast.

In the field of drug analysis volumetric methods of analyses, either direct or indirect, have their own importance due to their inherent simplicity. This is the reason why still a large number of official methods for the determination of pharmaceuticals in the pharmacopoeias are based on titrimetry. Though it is the oldest technique in the market now but still having their recognition in the field of scientific research.

Chromatographic methods have many applications in trace analysis and sometimes prove it as the only way. The different kind of it like paper chromatography, thin layer chromatography, column chromatography, capillary electrochromatography, high performance thin layer chromatography, high performance liquid chromatography and gas chromatography,

have most frequent applications in the field of pharmaceutical as well as the biomedical analyses. Thin layer chromatography (TLC) is a simple, in-expensive and rapid technique, having wide applications for the separation and identification of impurities and degraded products [5]. Capillary electrochromatography (CEC) is a method in which liquid mobile phase is driven through a stationary phase in a packed capillary column by the electro-osmotic flow generated by a large difference in potential across the column [6–9]. CEC previously has been utilised for the separation of certain pharmaceuticals; an interesting example is that of a diastereoisomeric mixture not successfully separated on a chiral HPLC column [10], which clearly shows its considerable advantage. Among all the chromatographic methods, high performance liquid chromatography is most acceptable nowadays due to its sensitivity and selectivity even in a multicomponent mixture. It is widely used for the identification of drugs in plasma and dosage forms [11–24], and creating its own position in the dissolution monographs of pharmacopoeias for the assay of drugs in dissolution fluids [25, 26].

Electrochemical methods are characterised by high sensitivity, selectivity and accuracy. These methods are based on the basic electrical parameters like current (I), resistance (R) and voltage (V) which has been utilised alone or in combination for analytical purposes. Pharmaceuticals containing phenolic, amino, heterocyclic nitrogen, ketonic or aldehyde group undergo oxidation at their characteristic potentials and hence selectivity of the technique is increased. Important electrochemical techniques such as amperometry, conductometry, potentiometry, anodic and cathodic stripping voltammetry, differential pulse voltammetry and polarography have been used in drug analysis [27]. Ion-selective electrodes are electrochemical sensors that allow potentiometric measurements of the activity of particular species in aqueous and mixed solvents, or partial pressures of dissolved gases in water [28].

Potentiometric titrations are also reported in the pharmacopoeias as the standard method for the determination of certain drug substances [29–31]. Despite the wide use of spectrometry and chromatography, analysts are choosing polarography and voltammetry due to their sensitive and characteristic qualitative as well as quantitative nature of analysis. Bersier has reviewed the importance of these techniques in pharmaceutical analysis [32].

Spectroscopic methods are widely used for the assay of drugs in pharmaceutical formulations. The most commonly used spectroscopic techniques for quantitative analysis are ultraviolet (UV) and visible methods. UV–spectrophotometry is best known and most widely applied in structure identification and quantitative analysis. It has become an established and fundamental technique in pharmaceutical research and analysis as well as in the broad area of organic and inorganic analysis. Conventional UV–spectrophotometric methods are simple, fast and economical and do not need any elaborate preparatory step for the samples prior to assay. Endriz [33] has described an UV–spectrophotometric method for the determination of heroin hydrochloride, methapyrilene hydrochloride, and quinine hydrochloride mixtures. The spectra for heroin, methapyrilene, and quinine show points of maximum absorption at 280 nm, 313 nm and 348 nm, respectively. Methapyrilene and quinine do not seriously interfere with the heroin maximum, and methapyrilene in dilute acid solution absorbs only slightly at the quinine maximum of 348 nm. The procedure is fast and accurate enough for forensic purposes. Analyses of chloramphenicol and tetracycline hydrochloride [34] and phenobarbital and pentobarbital in pharmaceutical formulations [35] are further examples of the utility of UV–spectrophotometry in this area. Because of the sensitivity of the UV method, the amount of sample required for the spectral identification is very small; drug levels and metabolites have been determined in biological tissues and fluids after separation and extraction. Since most

materials are not photosensitive, the technique is non-destructive, and the metabolite or drug can be recovered for further testing. However, these methods are inadequate in the presence of other components (like any drug of combination, excepients or decomposition products) showing similar UV spectra [36].

Photocolorimetric methods of analysis are performed in the visible region of light. They are based on the measurement of the absorbance of the coloured compounds. Usually, the analyte being colourless, they are reacted with the suitable chemical reagents in order to convert them in coloured compounds. Simple colorimetric and UV methods continue to be popular for carrying out single-component assays on a variety of formulated products. Representative examples of some of the many assays that have been published are given in Table 1.1.

In the course of this discussion, the importance of computer-aided spectrophotometric determination of multicomponent systems can not be ignored. The quantitative spectrophotometric analysis of active ingredients in a solution containing more than two substances can be difficult to achieve by means of the traditional methods. In order to resolve this problem, several papers were published on the application of least squares method and orthogonal polynomials for the analysis of multicomponent systems [64–69].

Impurities present on the particle surfaces, e.g. from residual mother liquor, may be low in terms of percentage but they may change in a significant way the behaviour of the powder in the manufacturing process or in the final medicinal product. Therefore, a methodology is needed to detect such deficiencies at the surfaces or in the upper layers of the powder. Spectroscopic reflectance technique of sufficient sensitivity and precision could be of

Table 1.1. Assay of drugs in pharmaceutical formulations by the spectrophotometric procedures.

Compound	Method	Conditions	Measurement (nm)	Reference
Acetaminophen	Colorimetric	By oxidative coupling with m-cresol	640	37
Ascorbic acid	Colorimetric	With 1-chloro 2,4-dinitrobenzene	380	38
Amlodipine besylate	Colorimetric	With p-chloranilic acid in chloroform-dioxane mixture	540	39
		With ninhydrin in DMF	595	40
		With bromothymol blue	405	41
		With 3-methyl 2-benzothiazolinone hydrazone hydrochloride	630	41
		With sodium hydroxide	456	42
Astemizol	Colorimetric	With p-chloranilic acid	540	43
		With suprachen Violet 3B	590	44
		With tropaeolin 000	500	44
		With iron(III) and 1,10-phenanthroline	515	44
Azapropazone	Colorimetric	With N-bromosuccinimide	488	45
		With N-chlorosuccinimide	451	45
Benidipine hydrochloride	UV	In methanol	238	46
Bromazepam	Colorimetric	With Mohr-salt	584	47
Diclofenac sodium	UV	In Tris buffer	284, 305	48
Diltiazem hydrochloride	Colorimetric	With sodium metavanadate in sulphuric acid	750	49
		With bromothymol blue	415	50

		With bromophenol blue	415	50
		With bromocresol green	415	50
Flurazepam	UV	In HCl solution	230	51
	UV-difference	Between pH 6 and pH 0	225	51
Loratadine	Colorimetric	With bromophenol blue	415	52
L- dopa	UV	With sodium hydroxide	300	53
Menadione	Colorimetric	With sodium hydroxide	450	54
Methyldopa	Colorimetric	With Ce(IV) nitrate in 2M sulphuric acid medium at 80 °C	550	55
		With barbituric acid at 100 °C	540	56
2-methyl 1,4- naphthoquinone	Colorimetric	With ethylacetoacetate and ethanolic ammonia	550	57
Nifedipine	Colorimetric	With potassium permanganate at neutral pH	530	58
Nalidixic acid	Colorimetric	With potassium persulphate in alkaline medium	390	59
Oxprenolol	Colorimetric	With cerium (IV) in sulphuric acid	480	60
Terfenadine	Colorimetric	With chromotrope 2B	530	61
		With chromotrope 2R	546	61
Thyroxin	Colorimetric	With nitrous acid in ice bath	420	62
Trimethoprim	UV	With potassium persulphate in alkaline medium	355	63

help to discriminate between apparently identical materials from different sources or to detect inconsistencies between the batches from the same source.

Near infrared (NIR) spectroscopy is a technique which has found its way into pharmaceutical control laboratories in recent years for raw material identification, water analysis and other pharmaceutical analyses [70–73]. Nuclear magnetic resonance (NMR) spectroscopy is a well-known spectroscopic technique specially used for the qualitative characterisation of the chemicals. In the recent attempts, NMR is also used as the sensitive quantitative method; a mini review has been presented in this context [74]. Moreover, NMR spectroscopy is favoured by chemists as a powerful technique for molecular structure determination.

Chemiluminescence is a powerful tool for drug analysis since its detection limits are extremely low, its instrumentation is very simple and of low cost. In combination with the derivatisation techniques in order to increase sensitivity, it has a wide range of applications. The excellent sensitivity and the versatility of the chemiluminometric methods of analysis are the main reason for the recent surge of interest in chemiluminescence. Certain drugs have been analysed based on the phenomena of chemiluminescence in liquid phase [75–77].

Flow injection analysis (FIA) is characterised by its simplicity, speed and the use of inexpensive equipment. In FIA, as a general principle [78], a quantity of accurately measured dissolved sample is injected or introduced into the carrier stream flowing through the system tubing, with or without additional changes like chemical reaction etc., occurring between the sample and the carrier. As the analyte (or its reaction product) passes through the continuous detector, a transient signal is generated and recorded. Therefore, FIA has certain clear

advantages like **(i)** reduced reagent consumption [79]; **(ii)** high sampling frequency [80]; **(iii)** safety in applying toxic reagents because the whole analysis proceeds in a closed system; and **(iv)** increased selectivity when the analyte is accompanied by more slowly reacting components. This technique has been utilised successfully in the determination of some compounds of pharmaceutical interest [27,81,82]. In the recent years, atomic absorption and emission spectrophotometry is also frequently used in the field of pharmaceutical analysis [83–85].

Difference spectrophotometry is an important and useful technique used in the determination of medicinal substances by eliminating specific interference from the degradation products, co-formulated drugs and non-specific irrelevant absorption from the formulation matrix. The technique involves reproducible alterations of the spectral properties of the absorbance difference between two solutions, provided that the absorbance of the other absorbing substance is not affected by the reagent(s) used to alter the spectral property [86].

Derivative spectrophotometry is an analytical technique of great utility for both qualitative and quantitative information from spectra composed of unresolved bands. Although it was introduced more than thirty years ago [87–89] and has advantages for the solution of specific analytical problems. The derivative method has found applications not only in the UV–visible spectrophotometry, but also in infrared [90], atomic absorption [91], flame emission spectrometry [92] and fluorimetry [93,94]. The use of derivative spectrometry is not restricted to special cases, but may be of advantage whenever quantitative study of normal spectra is difficult. Its disadvantage is that the differentiation degrades the signal-to-noise ratio, so that some form of smoothing is required in conjunction with the differentiation [95].

Even though many pharmaceuticals show native fluorescence, there is also an important group of compounds, which is not fluorescent, can be determined by using fluorescent probes through derivatisation reactions. An account of the fluorescent probes more widely used for the determination of drugs and related compounds have been reviewed [96].

In the recent period, analysts are much interested in coupling the chromatographic techniques with that of the spectroscopic techniques. The advent of liquid chromatography with mass spectrometry, gas chromatography with tandem mass spectrometry and liquid chromatography–electrospray tandem mass spectrometry are good examples. Hirsch *et. al.* have determined the antibiotics using chromatography–electrospray tandem mass spectrometry[97]. Dielectric relaxation spectroscopy and x–ray powder diffractometry are also utilised for the identification of pharmaceuticals [98,99]. The safety of a drug product is dependent not only on the toxicological properties of the active drug substance itself, but also on the impurities that it contains. Monitoring of drug substance's impurities is routinely accomplished using HPLC. However, HPLC retention times can vary, resulting in uncertainty as to whether a peak at a new retention time is a new impurity. Because standards of the minor impurities (less than 0.1% by area) are not usually available, some method is needed to characterise each of these peaks without isolating them. This on-line characterisation might be accomplished using UV diode array spectral matching [100].

Whenever the question of mathematical and statistical treatments arises, the role of chemometrics can not be ignored. Chemometrics is defined as *"the chemical discipline that uses mathematical, statistical and other methods of formal logic to design or select optimal procedures and experiments, and provide maximum chemical information by analysing chemical*

data" [101,102]. Chemometrics is the science that helps to make good use of information technology in the chemical analysis and to develop intelligent analysers, i.e. that automatically select the correct method for a given problem, carry it out, validate it and interpret the results.

Kinetic method of analysis, which is in fact, the study of the measurement of concentration changes (detected via signal changes in a reactant, which may be the analyte itself) with time after the sample and reagents have been mixed, is developing since late 1950s. They are not only the good choices for the drug analysis but very relevant to analytical chemistry in at least four respects: **(a)** it allows the elucidation of the physical, chemical and physico-chemical mechanisms on which analytical processes are based and hence their rational optimisation; **(b)** it facilitates the development of new analytical methods and techniques that are otherwise unattainable if the dynamic aspects are not dealt with; **(c)** it is the foundation of reaction rate methods (kinetic determinations); and **(d)** it contributes as sensitivity, selectivity and precision. The principles and applications of the kinetic methods have been reviewed [103–105]. Kinetic methods are generally rapid, reproducible and involve simple instrumentation. Though there are different modes of calibration in kinetic method, but the reaction rate methods involve two general aspects, which endow them with higher selectivity than equilibrium methods, namely the scarcity of parasitic blank signals and the possibility of using kinetic discrimination. In case of very fast reactions, the automatic handling is preferred for better and more accurate results; so the automation of the instrumentation is required. They can also be used to enhance the reproducibility and rapidity of slow reactions. Since kinetic methods of analysis require accurate timing, careful control over experimental conditions, precise sample and reagent preparation, proportioning and mixing, and accurate measurements of dynamic signals, hence they are well suited to intelligent automation. Kinetic

automatic techniques are generally based on open systems, among the most popular of which are stopped flow (SF) [106] system and the continuous addition of reagent (CAR) technique [107–110]. Several drugs have been determined by using the CAR technique with photometric [111,112] and fluorimetric detection [113]. On the other hand, the CAR technique has been extended to chemiluminescence (CL) reactions as CAR chemiluminescence spectrometry (CARCL), a new approach, which have proved outstanding for the analysis of drugs and other substances of analytical interest. The use of catalysts to accelerate analytical reactions is feasible with both reaction rate and equilibrium determinations. In this concern, the use of micellar media in kinetic methods is recently encouraged to enhance the rate of reaction (through micellar catalysis) which in turn reduces the time of analysis and also improves the sensitivity and selectivity [114–117]. Multicomponent kinetic determinations, often called as differential rate methods, are also receiving a good deal of current attention in research laboratories. The various approaches to multicomponent determinations have been reviewed recently by Perez–Bendito [118]. One new approach that has been proposed for dealing with overlapping spectra is the kinetic wavelength-pair method [119]. For a mixture of two components, the method relies on measuring the difference in the rate of change of absorbance with time at two pre-set wavelength pairs (four wavelengths). Another method called as the H–point standard addition method [120] proposed by Bosch–Reig *et. al.* is capable of kinetic determination of components with overlapping spectra in their binary mixture.

Pharmaceutical industry is one of the fast growing industries next to the information technology. Pharmaceutical analysis is a well-established section in the field of analytical chemistry whose advancements and works have been impressive in the last decades.

As mentioned above, the safety of drug products are dependent not only on the toxicological properties of the active drug substances itself but also on the impurities that it contains. Such impurities are associated with the drugs right from the manufacturing stages in the form of organic, inorganic and residual solvents (used to purify and generate the desired crystal morphology). The sources of such impurities may be the starting materials, intermediate and or synthetic by–products, reagents and catalysts. Furthermore, after getting the desired final product, the style of storage and class of packing may affect the stability of the drug substances, hence again leading to impurities, which raises a question mark on the recommended dosage amount supplied in the form of pharmaceutical formulations. Unlike other consumable products in the daily life, either the impurities or any abnormality in the recommended dosage amount of drug may lead to more adverse toxicological effects on the human life which may be life threatening.

The requirements of quality, quantity, purity and safety of pharmaceutical formulations warrants a careful thought by every one associated with this area. Due to easy availability of sub-standard drugs and medicines in the markets of the developing countries, it is crucial to assay drugs in dosage forms. Hence development of rapid, simple and cost-effective analytical methods for the analysis of drugs are the need of the day in public defence.

The increasing emphasis on the pharmacokinetic, bioavailability and therapeutic monitoring of drugs has placed a heavy burden on drug analysts. Reliable measurements of very low levels of drugs in complex matrices are frequently required. Because of the vast number of drugs introduced every year, more and more methods for drugs and metabolite determination are being developed either for routine or research use. Analytical procedures are

used throughout the drug development and the manufacturing of drug substances and drug products. Important decisions such as the establishment of the shelf-life from stability studies, the need for additional toxicological trials if new impurities appear or if known impurities exceed the qualified levels, the reworking of batches or batch release or rejection are based on analytical results. In order to make the right decisions and to avoid additional work, an appropriate performance of the analytical procedure is essential and this needs the *"suitability of the method for its intended use"*.

Once an analytical method is advent, it is important to validate the method before it should be recommended for the routine analysis. Method validation of analytical procedure, i.e. the proof of its suitability for the intended purpose, is an important component in determining the reliability and reproducibility of the methods and is required for any regulatory submission. The method should be defensible with respect to regulatory requirements and reliable by incorporating statistical analysis to evaluate its performance. Validation of analytical methods has been the subject of discussion in recent years [121–124]. The procedure of validation requires the calculation and fitting of the experimental data within a standard fixed criteria. Statistical techniques are used to evaluate the linearity, precision, accuracy, robustness/ruggedness, sensitivity (limit of detection and quantitation) and specificity etc. Other aspects needing to be considered include identification and handling of samples prior to analysis, stability of samples under various storage conditions and the continuous assessment (quality control) of the method during its routine use. Before an analytical method is used for routine analysis it must be demonstrated first that the method fulfils certain performance criteria, when this has been documented, the method is said to be validated.

There are several international organisations and regulatory authorities, which are involved, in fixing the criteria for the validation. Some of them are listed below (Table 1.2). In the field of drug analysis, it is very clear that the definitions cover the entire field of analytical chemistry from bioanalysis to substance and product analysis.

Table 1.2. Validation of analytical methods – international definitions.

Organisation	Applicability	Remarks
IUPAC	Worldwide	
ILAC	Worldwide	
WELAC	Europe	
ICH	Europe, Japan, USA	Only pharmaceutical products.
ISO	Worldwide	Lacks definitions of selectivity and specificity.

Abbreviations: **IUPAC**, International Union of Pure and Applied Chemistry; **ILAC**, International Laboratory Accreditation Conference; **WELAC**, Western European Laboratory Accreditation Co-operation; **ICH**, International Conference On Harmonisation; **ISO**, International Organisation for Standardisation.

It has been agreed that the key for evaluation of method reliability and overall performance are: **(i)** analyte stability, **(ii)** method selectivity/ specificity, **(iii)** limit of detection and quantitation, **(iv)** accuracy and precision, **(v)** relationship between the response and concentration, **(vi)** recovery and **(vii)** ruggedness. The overall validation strategy consists of four components, which are prevalidation, validation proper, study proper and statistical analysis.

Prevalidation

Prevalidation requires the availability of an authenticated analytical reference standard to prepare solutions of known concentrations. This standard should be of known form, e.g. free

base or salt and of known purity, if used over a time, should be monitored to ensure no decomposition or contamination has taken place. Prevalidation provides the analyst an opportunity to obtain some practical experience with the method and helps to identify the optimum experimental conditions. It is also recommended that the following studies be conducted prior to initiating the validation proper. The appropriate measurements of concentration changes (detected via signal changes) in a reactant (which may be the analyte itself) with time after the sample and the reagents have been mixed. The optimum standard curve range and the number of calibrators should be established. The appropriate regression models which best fit the data is then selected. The extraction scheme and its recovery should be optimised to give insight into the limit of quantitation.

Sampling and storage is an important initial part during the analytical studies. The quality of analytical data depends critically on the validity of the sample and the adequacy of the sampling procedure. Drugs may be lost to the container or be degraded chemically, photochemically or enzymetically during storage. Hence, an investigation of the stability of the drug during sample storage (temperature and duration) is a crucial part of the method validation. The lack of information on stability may jeopardise subsequent investigations. In general, the samples that will be analysed within a few days should be stored at 4 °C; those that are to be stored for longer should be kept at −20 °C. For very long-term storage, freeze-drying should be considered. Provided it is available in sufficient quantity, the sample should be divided into aliquots before freezing, to minimise precipitation or degradation due to repeated freezing and thawing. Stock solutions of photodegradable drugs should be stored in amber-coloured containers.

Validation proper

Among all of the international organisations, the ICH guidelines achieved a great deal in harmonising the definitions of the required validation characteristics and their basic requirements. The International Conference on the Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human Use has harmonised the requirements in two guidelines [125,126]. The first one summarises and defines the validation characteristics needed for various types of test procedures; the second one extends the previous text to include the experimental data required and some statistical interpretation. These guidelines serve as a basis worldwide both for regulatory authorities and industry and bring the importance of a proper validation to the attention of all those involved in the process of submission. In order to fulfil the validation responsibilities properly, the background of the validation parameters and their consequences must be understood. Normally evaluated validation characteristics and their minimum number of determinations (if applicable) are listed in Table 1.3 [126].

There has been controversy regarding the technical term for the validation characteristic, i.e. specificity vs. selectivity, and the current definition of ICH is not clear in this respect. A clear distinction must be made between the terms *"**specific**"* and *"**selective**"*. A specific reaction or test is one that occurs only with the substance of interest, while a selective reaction or test is one that can occur with other substances but exhibits a degree of preference for the substance of interest. Few reactions are specific, but many exhibit selectivity. WELAC refers the selectivity of a method to the extent to which it can determine particular analyte(s) in a complex mixture without interference from other components in the mixture. A method which

Table 1.3. Validation characteristics normally evaluated for the different types of procedures [125] and the minimum number of determinations required (if applicable) [126].

Validation Parameters	Minimum number	Test procedure			
		Identity	Impurities		Assay[a]
			Quantitative	Limit	
Specificity[b]		Yes	Yes	Yes	Yes
Linearity	5 concentrations	No	Yes	No	Yes
Range		No	Yes	No	Yes
Accuracy	9 determinations over 3 concentrations (e.g. 3x3)	No	Yes	No	Yes
Precision					
Repeatability	6 determinations at 100% or 9 determinations over 3 concentration levels (e.g. 3x3).	No	Yes	No	Yes
Intermediate Precision	2 series	No	Yes	No	Yes
Reproducibility[c]					
Detection limit		No	No[d]	Yes	No
Quantitation limit		No	Yes	No	No

[a] Including dissolution, content potency.

[b] Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

[c] Intermediate precision sufficient for submission.

[d] May be needed in some cases.

is perfectly selective for an analyte or a group of analytes is said to be specific. Thus a specific test procedure measures quantitatively a chemical or a physical parameter of functional group of one or more different analytes in the sample matrix while a selective one detect qualitatively the analyte in the presence of components which may be expected to be present in the sample matrix. Fig. 1.3 gives a graphical presentation for the differentiation between selectivity and specificity.

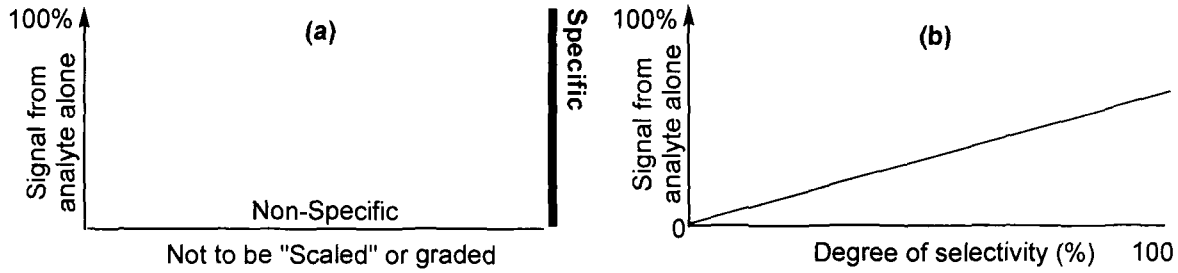


Fig. 1.3. Graphical demonstration of selectivity and specificity. The percentage of the measured signal attributable to the analyte alone (on the *Y*-axis) is given as a function of the degree of selectivity **(b)**. A perfectly selective method is said to be specific **(a)**.

Calibration range is defined by the expected concentration in the samples, and will be usually in the linear range of the detector for that analyte, where the determined response is directly proportional to the concentration The calibration range should neither be too wide nor too narrow [122]. Least square linear regression is normally be used to define the calibration line mathematically. The range of an assay method can also be defined as the concentration intervals over which an analyte can be measured with acceptable precision and accuracy, wherease the linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample [125]. It has been generally accepted that the coefficient of correlation of the line of regression should exceed 0.99 [127]. However, there are certain analytical procedures (TLC,

fluorescence and atomic absorption spectrometry) with non-linear responses, which raises the question about the suitability of the calibration mode to be used in the procedure. The requirements and relevant parameters for the various calibrations are given in Table 1.4.

Table 1.4. Requirements for the different calibration modes with relevant parameters.

Quantitation	Requirements	Relevant parameters
Single Point Calibration External standard	Linear Function	Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).
	Non-significant ordinate intercept	Inclusion of zero in confidence interval of the ordinate intercept, magnitude of the intercept (as percent of the signal at 100% test concentration).
	Homogeneity of variances[a]	*F*–test of the variances at the lower and upper limits of the range.
Multiple Point Calibration Linear, Unweighted	Linear function	Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).
	Homogeneity of variances[a]	*F*–test of the variances at the lower and upper limits of the range.
Linear, Weighted	Linear function	Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).
Non-linear	Continuous function	Appropriate equation
100%–method (area normalisation for impurities)	For main peak: Linear function	Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).
	Non-significant ordinate intercept	Inclusion of zero in confidence interval of the ordinate intercept, magnitude of the intercept (as percent of the signal at 100% test concentration).
	Homogeneity of variances[a]	*F*–test of the variances at the lower and upper limits of the range.
	For impurities: Linear function	Standard error of slope (residual standard deviation), sensitivities (RSD, graph), residual analysis, statistical tests (vs. quadratic regression).

[a] May be presumed for a limited range (factor 10 – 20).

Bias is the difference between the expectation of the results and an accepted reference value. Bias is a systematic error (determinate error) contrast to the random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the accepted reference value is reflected by larger bias value. Calibration curves are accepted for each individual batch during validation when no single standard has a % bias greater than 20% [122]. When a new analytical method is to be tested in a laboratory it may be used on samples of suitable reference material, but more often it is compared with an existing method on a range of suitable materials whose concentration levels are not known at all, precisely. It is important that the samples chosen cover the range of concentrations expected in future use, as the bias of the new method can vary with concentration. Regression techniques are commonly used to estimate the bias of the new method [128].

Accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value, and the value found [125]. The ICH guideline recommends the demonstration of accuracy over the whole working range. However, if only a narrow range is required (e.g. assay or impurities with a low specification limit), a six fold determination at a 100% test concentration may also be used. A quantitative approach to demonstrate the accuracy according to ICH guidelines have been given in Table 1.5. The analytical test to be validated is compared with another procedure or applied to a reference substance, the probably different specificities must be taken into account. Therefore, statistical tests should be performed only if the systematic bias based on these differences can be quantified and thus are corrected or are negligible. Otherwise the comparison should be performed as a qualitative verification of plausibility or an acceptable

maximum difference should be defined. The accuracy can be established in different ways, using statistical analysis.

Table 1.5. Quantitative approaches to demonstrate accuracy according to ICH [126].

Drug substance	Application of the analytical procedure to a reference material. Comparison of the results with those of a second well characterised procedure.
Drug product	Application of the analytical procedure to synthetic mixtures of drug product components Spiking of analyte to drug product. Comparison of the results with those of a second well characterised procedure.
Impurities (quantitative)	Spiking of the impurity to drug substance or product. Comparison of the results with those of a second well characterised procedure.

Precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed condition [125]. Precision should be measured using homogenous authentic samples. However, if it is not possible or if it is not practical to obtain a homogeneous sample, it may be measured using artificially prepared samples or sample solutions. Precision experiments study the effect of random variations on the performance of a method. Precision must be assessed in several ways:

Repeatability, also termed as intra-assay precision, refers to the precision obtained under the same operating conditions over a short interval of time by applying the whole

analytical procedure to the sample. Repeatability should be assessed using a minimum of 6 determinations at 100% of the test concentration or a minimum of 9 determinations over the range of the procedure (e.g. 3 replicates for each of 3 concentrations). This will provide an estimate of the random error of the method.

Intermediate precision refers to within-laboratory variations, preferably performing a method over an extended period of time. The extent of investigation will depend on the intended use of the procedure. A typical investigation of intermediate precision would study various parameters that might include analysts, days, equipment, reagents, columns, etc. It is not required that each effect be studied individually so, therefore, experimental design is encouraged.

Reproducibility is an estimate of the variation between different laboratories and is usually assessed by performing an inter-laboratory (collaborative) study. A study of reproducibility is not required as a part of the marketing authorisation dossier.

Detection limit (DL) is the lowest amount of analyte that can be detected, as it (the analyte) yields instrumental response greater than a blank, but can not be quantified. It is a parameter of *"limit test"* and expected to produce a response, which is significantly different from that of a blank. On the other hand, quantitation limit (QL) is a parameter of *"determination test"* and can be defined as the lowest concentration of analyte that can be measured and quantified with acceptable precision and accuracy. Hence, it is the lowest concentration included in the standard curve and is used to interpolate unknown sample concentration. One should be aware that the determined QL (or DL) is strongly related to the equipment actually used, as well as to the time of determination. They may represent more

system parameters than characteristics of the analytical procedure. In cases where a general QL is required, as in pharmaceutical analysis, it is essential to define a realistic QL (or DL) for the analytical procedure, independently from the equipment used, because this limit has important consequences (e.g. for the reporting threshold for impurities or for method transfer).

DL and QL can be calculated from the standard deviation of the blanks. This approach is usually performed with instrumental methods. Measuring a blank sample, the standard deviation is calculated from the numerical outputs. DL and QL are defined as the 3.3 and 10 fold, respectively, of the standard deviation of the blank. The values are converted to a concentration by the slope of a corresponding calibration line [126].

Robustness/ruggedness of an analytical method can be defined as *"a measure of the capacity of analytical procedure to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage"* [129]. It can also be described as the ability to reproduce the (analytical) method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method. Though these two terms have been frequently used as the synonyms [130] but sometimes a mutual differentiation have also been made [131], according to which the ruggedness is defined as the degree of reproducibility of the test results obtained under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days, etc. The ICH guidelines also recommend that *"one consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g.*

resolution tests) is established to ensure that the validity of the analytical procedure is maintained whenever used" [129]. Deliberate variations in the parameters of a procedure will provide an estimate of its reliability in routine use. The extent of robustness studies will depend upon the type of method but this evaluation should be considered during the development phase. Typical variations might include extraction time, flow rate through a testing device, stability of test solutions, etc.

Study Proper and Statistical Analysis

Daily standard curves are generated to determine the sample concentrations. The quality control sample sequence is carefully monitored for the systematic errors. For each standard curve, the slope, the intercept, variance and correlation coefficient should be reported. From the data generated, specific analytical parameters should be reported including linearity, accuracy, precision, sensitivity and recovery. Acceptance of the assay results is determined by monitoring the quality control results. If the concentrations are within the control chart's confidence limits established during the method validation, the data are considered valid. Upon completing a study proper and accepting the analytical runs, the quality control results are incorporated into their respective databases to update their confidence limits.

Statistical calculations of the validation parameters and certain others of common interest

Linear regression by least square method; Normalisation factor (unweighted linear regression: $w_i = 1$, $k_w = 1$):

$$k_w = \frac{n}{\sum(w_i)}$$

Means:

$$\bar{x} = \frac{\sum(x_i * k_w * w_i)}{n} \qquad \bar{y} = \frac{\sum(y_i * k_w * w_i)}{n}$$

Sum of squares:

$$Q_{xx} = \sum(k_w * w_i * (x_i - \bar{x})^2) \qquad Q_{yy} = \sum(k_w * w_i * (y_i - \bar{y})^2)$$

$$Q_{xy} = \sum(k_w * w_i * (x_i - \bar{x}) * (y_i - \bar{y}))$$

Residual sum of squares (Q_{yy}) and Residual standard deviation (s_y):

$$Q_{yy} - \frac{Q_{xy}^2}{Q_{xx}} \qquad s_y = \sqrt{\frac{Q_{yy} - \frac{Q_{xy}^2}{Q_{xx}}}{n-2}}$$

Slope (b), standard deviation of slope (s_b) and Relative confidence interval of the slope (CI_b):

$$b = \frac{Q_{xy}}{Q_{xx}} \qquad s_b = \sqrt{\frac{s_y^2}{Q_{xx}}} \qquad CI_b = \frac{100 * t(P, n-2) * \sqrt{s_b^2}}{b}[\%]$$

Intercept (a), standard deviation of intercept (s_a) and Confidence interval of the intercept (CL_a; upper and lower limits):

$$a = \bar{y} - b * \bar{x} \qquad s_a = \sqrt{s_y^2 * \left(\frac{1}{n} + \frac{\bar{x}^2}{Q_{xx}}\right)} \qquad CL_a = a \pm t(P, n-2) * \sqrt{s_a^2}$$

Standard error (s_{x0}) and Relative standard error of slope (V_{x0}):

$$s_{x0} = \frac{\sqrt{s_y^2}}{b} \qquad V_{x0} = 100 * \frac{s_{x0}}{\bar{x}}[\%]$$

Coefficient of correlation (r):

$$r = \frac{Q_{xy}}{\sqrt{Q_{xx} * Q_{yy}}}$$

Residuals and %-Difference:

$$y_i - \hat{y}(x_i) \qquad \frac{(y_i - \hat{y}(x_i))}{\hat{y}(x_i)} * 100[\%]$$

Where y_i = experimental *y*–value and $y(x_i)$ = calculated *y*–value for the given *x*–value.

Calculation of concentrations with uncertainties:

Input of *y*–values: Calculation of concentration $X_i(y_i) = \frac{(y_i - a)}{b}$

Input of *x*–values: Calculation of signal $y_i = a + b * x_i$

Uncertainty and Relative uncertainty:

$$\pm \frac{t(P\%, n-2)*s_y}{b} * \sqrt{\frac{1}{n} + \frac{1}{m} + \frac{(y_i - \bar{y})^2}{b^2 * Q_{xx}}} \qquad \pm \frac{100\% * t(P\%, n-2)*s_y}{b * X_i(y_i)} * \sqrt{\frac{1}{n} + \frac{1}{m} + \frac{(y_i - \bar{y})^2}{b^2 * Q_{xx}}}$$

Accuracy

(1) by comparison and validation procedure:

Mean (*x*), $\bar{x} = \frac{1}{n} * \sum_{i=1}^{n} x_i$

Confidence interval ($CL(\bar{x})$ upper and lower limit),

$$CL(\bar{x}) = \bar{x} \pm s * \frac{t(P, n-1)}{\sqrt{n}}$$

Variance (s^2), Standard deviation (**s**) and Coefficient of variation (*CV* or *RSD*),

$$s^2 = \frac{\sum (x_i - \bar{x})^2}{n-1} \qquad \mathbf{s} = \sqrt{\mathbf{s}^2} \qquad CV = \frac{s}{\bar{x}} * 100$$

Range [R%], $R = \frac{x_{max} - x_{min}}{\bar{x}} * 100$

Precision (Analysis of variance study):

(1) Test for the homogeneity of variances according to Cochran. $C = \frac{s^2_{max}}{\sum_{j=1}^{k} s_j^2}$

If > $Cr(P, n-1)$; variances are inhomogeneous. $k_{max} = 10$, $n_{max} = 10$

Statistical linearity test

As statistically based linearity test, the significance of the quadratic coefficient can be checked. If the confidence interval of the quadratic coefficient c ($y = a + b*x + c*x2$)

includes zero, the quadratic term becomes neglectable and the equation is reduced to a linear function (equations).

Significance of the quadratic coefficient: $CI_c = t(P, n-3) * \sqrt{s_c^2}$

CI_c = confidence interval of the quadratic coefficient.

If $c \pm CI_c$ includes 0, quadratic coefficient is not significant, and hence, no significant better fit by quadratic regression.

The test according to Mandel is also based on a comparison of a linear and a quadratic response function. In this case, a test is performed to determine whether the quadratic function results in a significantly better fit by comparing the residual variances of both calibrations. If $PG = \frac{(n-2) * s_{y1}^2 - (n-3) * s_{y2}^2}{s_{y2}^2}$

$> F(P, F_1 = 1, F2 = n - 3)$; significant better fit by quadratic regression. Where s^2_{y1} is residual variance linear regression and s^2_{y2} is residual variance linear regression.

Linearity (overall analysis, unweighted linear regression)

Comparison with the slope of the 1st series:

Limits of the equivalence interval:

$$C_U = 100\left\{\left(\frac{b_j}{b_1}\right)\exp\left[s_r\, t\left(P, n_1 + n_j - 4\right)\right] - 1\right\} \quad C_L = 100\left\{\left(\frac{b_j}{b_1}\right)\exp\left[-s_r\, t\left(P, n_1 + n_j - 4\right)\right] - 1\right\}$$

$\delta\%$ = Acceptable difference (percentage).

$t(P,f)$ = Student's t– value for level of statistical confidence P and degrees of freedom, $f = n_i + n_j - 4$.

Where $b_{1,j}$, $sb_{1,j}$, $n_{1,j}$ = slope, standard deviation of the slope, and number of values of series 1 and j, respectively.

The slopes of series 1 and j are equivalent, if – $\delta\% \leq C_L \wedge C_U \leq \delta\%$

Accuracy (recovery from linearity): Comparison of the slope with the theoretical value of *1*.

Limits of the Equivalence interval:

$$C_L = b - 1 - t(P, n-2)\frac{s_y}{\sqrt{Q_{xx}}} \qquad C_U = b - 1 + t(P, n-2)\frac{s_y}{\sqrt{Q_{xx}}}$$

δ = acceptable difference (absolute value).

t(P,f) = Student's *t*–value for level of statistical confidence *P* and degrees of freedom, $f=n-2$.

s_y, Q_{xx} = residual standard deviation, sum of squares.

The slope is equivalent to the theoretical value of *1*, if $-\delta \leq C_L \wedge C_U \leq \delta$

Accuracy (100% recovery, comparison to reference): Comparison of the mean with a nominal value.

Limits of the equivalence interval:

$$C_U = T - \bar{x} + t(P, n-1)\frac{s}{\sqrt{n}} \qquad C_L = T - \bar{x} - t(P, n-1)\frac{s}{\sqrt{n}}$$

T = nominal value (100% or reference value).

δ = Acceptable difference (absolute value).

t(P,f) =Student's *t*–value for level of statistical confidence *P* and degrees of freedom, $f = n-1$.

Where $\bar{x}, s, n$ = mean, standard deviation and number of values, respectively.

T are equivalent, if $-\delta \leq C_L \wedge C_U \leq \delta$

Accuracy (Comparison to another procedure): Comparison of two means

$$C_L = 100\left\{\left(\frac{\bar{x}_1}{\bar{x}_2}\right)\exp\left[-t(P, n_1+n_2-2)s\right]-1\right\} \qquad C_U = 100\left\{\left(\frac{\bar{x}_1}{\bar{x}_2}\right)\exp\left[t(P, n_1+n_2-2)s\right]-1\right\}$$

$$s = \sqrt{s_p^{\,2} * \left(\frac{1}{n_1 * \bar{x}_1^{\,2}} + \frac{1}{n_2 * \bar{x}_2^{\,2}}\right)} \qquad s_p^{\,2} = \frac{(n_1-1)*s_1^{\,2} + (n_2-1)*s_2^{\,2}}{n_1+n_2-2}$$

δ% = Acceptable difference (percentage).

t(P,f) = Student's *t*–value for level of statistical confidence *P* and degrees of freedom, $f=n_1+n_2-2$.

Where $\bar{x}_{L2}, s_{L2}^2, n_{L2}$ =mean, variance, and number of values of series 1 and 2, respectively.

The two means are equivalent, if $-\delta\% \leq C_L \wedge C_U \leq \delta\%$

Precision (individual series): Comparison of the standard deviation with a nominal value.

Limits for the equivalence interval:

$$C_L = 100\left\{\frac{s}{T}\sqrt{\frac{n-1}{\chi^2(P, n-1)}} - 1\right\}, C_U = 100\left\{\frac{s}{T}\sqrt{\frac{n-1}{\chi^2(1-P, n-1)}} - 1\right\}$$

$\delta\%$ = Acceptable difference (percentage of the standard deviation).

$\chi^2(P, f)$ = Chi-squared distribution for level of statistical confidence P or $1 - P$ and degrees of freedom $f = n - 1$.

$\bar{x}, s^2, n$ = mean, variance, and number of values.

Experimental and target standard deviation are equivalent, if $-\delta\% \leq C_L \wedge C_U \leq \delta\%$

Test for Outliers

With regard to linearity, an outlier is characterised by a significant deviation from the regression curve. The 95% prediction interval can be used as a criterion [132]. This interval describes the range around the regression function in which a repeated analytical value can be expected in 19 out of 20 cases. If the calculation of the 95% prediction interval is performed with all values, a possible outlier might have a considerable influence on the calculation (by broadening the interval). The suspected value or values can be inactivated manually and a second regression is performed. If the deactivated value or values are now outside the 95% prediction interval, they can be suspected as being outliers. In the same manner, deviations from a linear response performing a linear regression can be detected. However, it must be taken into consideration that the prediction interval will be less stringent (broader) as the

extrapolation increases ("trumpet-shaped"). Most statistical tests and calculations are based on the assumption that the experimental values are only influenced by random variability (i.e. that they are normally distributed). Data that do not fulfil these assumptions (e.g. due to so called "gross errors", weighing, dilution, or by problems with the instrument etc.) will affect the results. It is the aim of outlier tests to identify such values in order to eliminate them before performing further calculations. However, the problem is especially with a small number of data where groupings could easily occur avoiding the incorrect rejection of values belonging to the same distribution. Therefore, outlier tests should be applied carefully and only obviously deviating values should be eliminated. When an outlier is identified, the absolute magnitude of the coefficient of variation (relative standard deviation) must also be considered for evaluation. If this parameter (calculated including the suspected "outlier") lies in a normally expected range, preferably all values should be retained. The question of systematic effects influencing the values (e.g. degradation or adsorption of the analyte, shifts in the equipment settings, etc.) is of more importance. This can be investigated by applying a trend test in which an unweighted linear regression is performed using the values in the order of the input (equidistant spacing with respect to the x dimension). The 95% confidence interval of the slope is then tested against the slope itself. If a slope can not be detected statistically, the hypothesis of a systematical trend can be rejected.

Unweighted linear regression of $i = 1$ to n vs. x_i. If $CI_b > abs(b)$ then no trend.

Where CI_b = confidence interval of the slope $t(P, n - 2)$ = Student's t–value for the level of statistical confidence P and degrees of freedom $n - 2$.

The outlier test according to Grubbs calculates the ratio of the difference between the largest/smallest value and the mean to the standard deviation. This parameter is then compared with the tabulated critical values. The test for double outliers searches for the occurrence of two outlying results.

$n_{max} = 50$. Range of the values x_i from *1* to *n*.

$$PG_{max} = \frac{(x_n - \bar{x})}{s} \qquad PG_{min} = \frac{(\bar{x} - x_1)}{s}$$

Test for single outliers:

$\bar{x}$ = mean, s = standard deviation.

An outlier may be suspected if *PG* is larger than the tabulated value [133].

Test for double outliers: $$d_0^2 = \sum_{i=1}^{n} (x_i - \bar{x})^2$$

Test of the two smallest values:

$$PG = d_{1,2}^2 / d_0^2 \qquad d_{1,2}^2 = \sum_{i=3}^{n} (x_i - \bar{x}_{1,2})^2 \qquad \bar{x}_{1,2} = \frac{1}{n-2} \sum_{i=3}^{n} (x_i)$$

Test of the two smallest values

$$PG = d_{1,2}^2 / d_0^2 \qquad d_{1,2}^2 = \sum_{i=3}^{n} (x_i - \bar{x}_{1,2})^2 \qquad \bar{x}_{1,2} = \frac{1}{n-2} \sum_{i=3}^{n} (x_i)$$

An outlier may be suspected if *PG* is smaller than the tabulated value [133].

For the outlier test according to Dixon [134], differences between ordered values at the upper and lower extremes are divided by the whole range. This parameter is then compared with the critical tabulated values.

$n_{max} = 40$. Range of the value x_i from *1* to *n*.

For $n = 3 - 7$

$$PG = \frac{x_2 - x_1}{x_n - x_1} \qquad PG = \frac{x_n - x_{n-1}}{x_n - x_1}$$

or

For $n = 8 - 12$: < *BR* >

$$PG = \frac{x_2 - x_1}{x_{n-1} - x_1} \qquad PG = \frac{x_n - x_{n-1}}{x_n - x_2}$$

or

For $n = 13 - 40$: < *BR* >

$$PG = \frac{x_3 - x_1}{x_{n-2} - x_1} \qquad PG = \frac{x_n - x_{n-2}}{x_n - x_3}$$

An outlier may be suspected if *PG* is larger than the tabulated value [135].

Test for normal distribution (David's test)

For all statistical tests used here, it is assumed that the experimental values are only influenced by random variability (i.e. that they are normally distributed). This can be checked with a statistical test according to David [136]. For this test, the ratio of the range (maximum to minimum value) to the standard deviation is compared with the tabulated critical limits. Relying on only a small number of data, the test for normal distribution is restricted to similar problems as discussed for the outlier tests. For a large number of analytical procedures, a normal distribution of the whole population can be generally assumed. The question of systematic effects influencing the values (e.g. degradation or adsorption of the analyte, shifts in the equipment settings, etc.), is of more importance. This can be investigated by applying a trend test.

R = range (maximum value – minimum value)

s = standard deviation

The values are (probably) normally distributed if $G_u < R/s < G_o$

G_u, G_o: lower and upper test limit. Tabulated value [136].

F- and t-tests:

(i) Mean *t*-tests:

F-test: If $\frac{s_1^2}{s_2^2} > F(P, f1, f2)$ with $s_1^2 > s_2^2$ then significant difference of variances exists. *F(P, f1, f2)* = Fisher's *F*-value for level of statistical confidence *P* and degrees of freedom f_1 and f_2.

If $\frac{D_j}{s_d} * \sqrt{\frac{n_j * n_{comparison}}{n_j + n_{comparison}}} > t(P, f)$ significant difference of mean exists.

Where $s_d = \sqrt{\frac{(n_j - 1) * s_j^2 + (n_{comparison} - 1) * s_{comparison}^2}{f}}$ $D_j = |\bar{x}_j - \bar{x}_{comparison}|$

(ii) Nominal value *t*-test:

If $\frac{D_j}{s_j} * \sqrt{n_j} > t(P, f)$ significant difference to the reference exits.

Where $f = n_j - 1$ and $D_j = |\bar{x}_j - x_{reference}|$

t(P, f) = Student's *t*-value for level of statistical confidence *P* and degrees of freedom *f*.

and $\bar{x}, s, n_j$ = mean, standard deviation, number of values in validation series.

CLASSIFICATION OF DRUGS

All the drugs according to their chemical nature can be divided into organic and inorganic compounds. They can be prepared synthetically (from chemicals) or can be directly obtained or reconstituted from the natural sources/products. All the drugs having medicinal importance can be broadly divided into two classes.

Chemical classification: Here the drugs are classified according to their chemical structure and properties without taking the pharmacological actions under consideration. In this

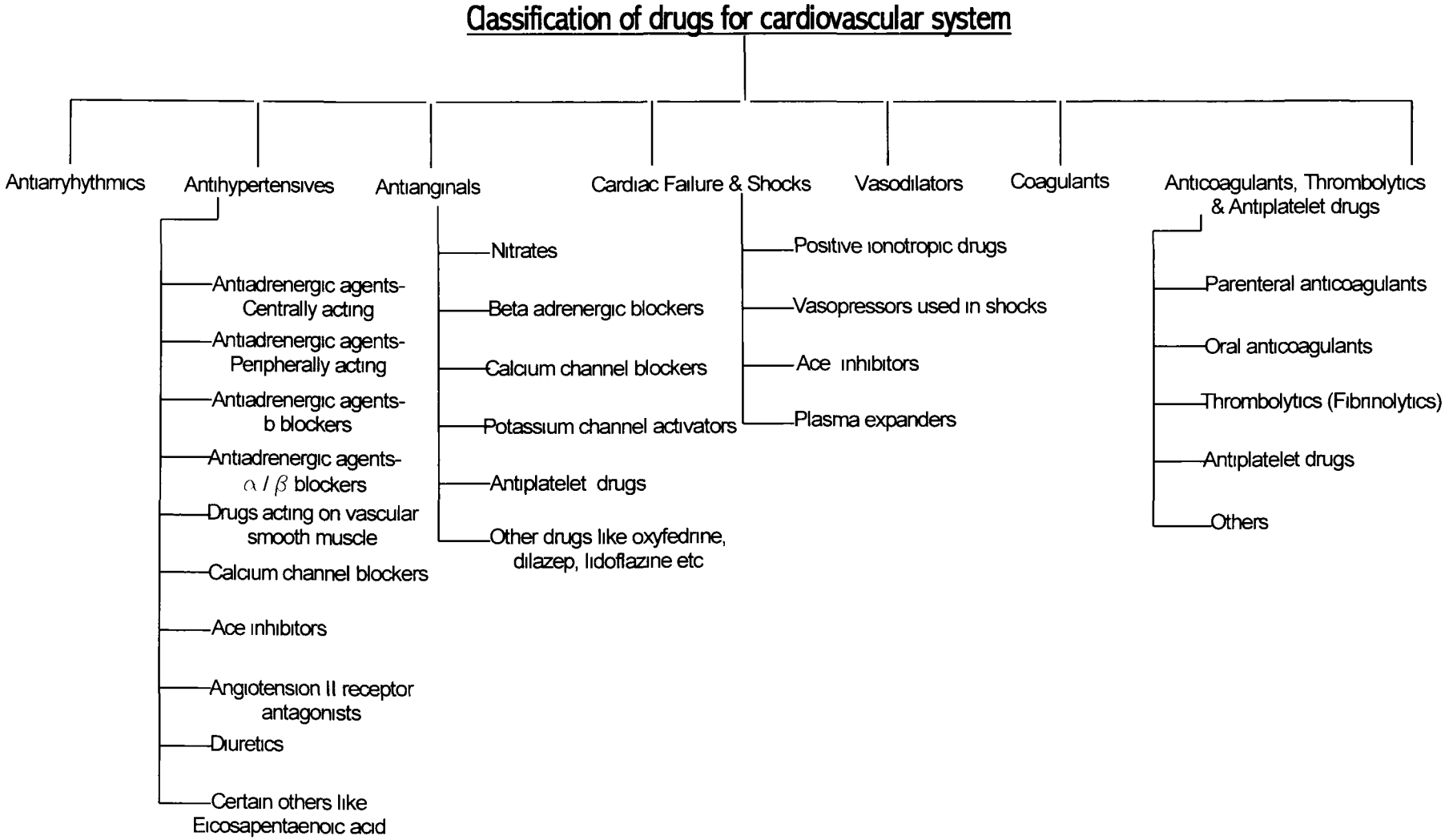
class most of the drugs are having at least an organic substrate, the further classification is done in the relevant manner.

Pharmacological classification: In this class the drugs are divided according to their action on the organism/organ (viz. heart, brain, lymphatic system, respiratory system, endocrine system, central nervous system etc.). Hence these drugs are named like antianginal, narcotics, soporifics, analgesics, diuretics and anaesthetics etc. Further classification of each group is done according to the therapeutic/pharmacological specificity with the relevant organ. A detailed classification of drugs with respect to their therapeutic importance for the cardiovascular system has been given in Table 1.6 [137].

ANTIANGINALS

Angina pectoris is a heart ailment characterised by pressing chest pain that often radiates to the neck area and arms and shoulders (often towards left side). As a clinical entity it was first characterised under the name *Pectoris Dolor* by William Heberden in 1768, although its symptoms had been noted as early as 1632 [138–140]. In the recent medical terminology it is referred as the symptom of ischaemic heart disease. For nearly a century following its first description, there was not much that could be done to relieve an angina patient of his agony; brandy, ether, chloroform, ammonia and other stimulants and depressants had been tried, but nothing seem to bring comfort. The breakthrough came in 1867 when **T. Lauder Brunton**, a British physician, reported his success with amyl nitrite. In the authoritative but lively journal of medicine, the *Lancet,* he wrote, *" On pouring from five to ten drops of (amyl) nitrite on a cloth and giving it to the patient to inhale, the pain completely disappeared and, generally did not return till its wonted time next night"* [141]. And finally the continuous effort of the medical

Table 1.6. Classification of drugs used in the treatment of cardiovascular disorders.



researchers, they got succeeded in knowing the different reasons with this life-threatening ailment and its therapeutic solutions.

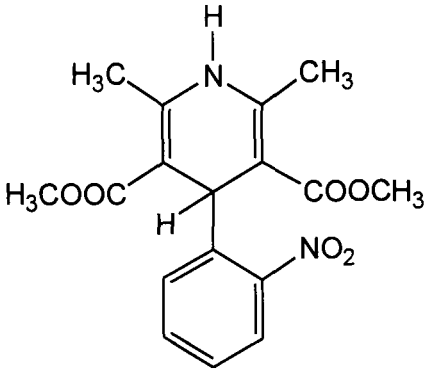
Drugs used in angina pectoris are those that reduce cardiac work and myocardial need by **(a)** unloading the heart, **(b)** dilating capacitance and resistance vessels, **(c)** dilating coronary arteries and **(d)** blocking ß– adrenoceptors. Anginal pain occurs when the coronary blood flow is insufficient to meet the heart's metabolic requirements. The drugs that either improve myocardial perfusion or reduce the metabolic demand, or both can counteract it. Several antianginal drugs are available for the treatment of angina. These include **(a)** organic nitrates, **(b)** beta-adrenergic blockers, **(c)** calcium channel blockers, **(d)** potassium channel activators, **(e)** antiplatelet drugs and **(f)** certain others like diazepam, oxyfedrine etc.

This thesis deals with the quantitative analysis of certain antianginal drugs namely, nifedipine, verapamil hydrochloride, amlodipine besylate and diltiazem hydrochloride in pharmaceutical formulations.

Nifedipine ($C_{17}H_{18}N_2O_6$)

Nifedipine is chemically dimethyl–1,4–dihydro–2,6–dimethyl–4–(2–nitrophenyl) pyridine–3,5–dicarboxylate promising for the following structure (Structure I). It is referred as the prototype compound of dihydropyridine class of calcium channel blockers having properties of peripheral and coronary vasodilation. It is orally administered for the management of hypertension and angina pectoris and functions primarily through its vasodilating properties as an afterload reducer.

Since nifedipine is extremely sensitive to photo-oxidation, manufacturers take great care in the storage and packaging of nifedipine to protect it from photodegradation due to

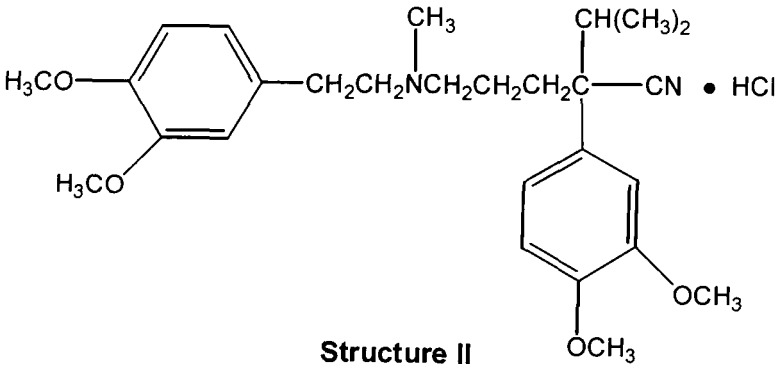


Structure I

inadvertent light exposure and thus to maintain its therapeutic potency. But due to the same reason, official United States Pharmacopoeia recommend that the measured amount of nifedipine in commercial preparations may vary in the range 90—110% of the labelled quantity.

Verapamil hydrochloride ($C_{27}H_{38}N_2O_4$. HCl)

Verapamil hydrochloride, chemically designated as 5—[N—(3,4—dimethoxyphenylethyl) methylamino]—2—(3,4—dimethoxyphenyl)—2—isopropyl valeronitrile monohydrochloride is an antiarrhythmic as well as a calcium channel blocker belonging to the phenylalkylamine class of compound. The following structure (Structure II) has been suggested for verapamil hydrochloride.



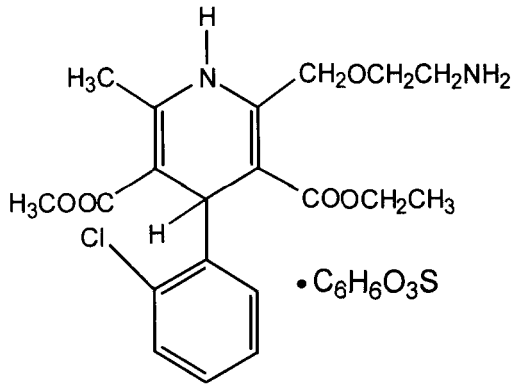
Structure II

Verapamil is administered orally as the racemic mixture and well absorbed rapidly. Verapamil is metabolised stereoselectively, the more active enantiomer being more rapidly

metabolised. According to pharmacopoeial definitions, it contains not less than 99.0% and not more than 101.0% of $C_{27}H_{38}N_2O_4$. HCl, calculated with respect to the dried substance.

Amlodipine besylate ($C_{20}H_{25}ClN_2O_5$. $C_6H_6O_3S$)

Amlodipine besylate is comparatively a new calcium channel blocker, and is chemically defined as 3–ethyl 5–methyl 2–(2–aminoethoxymethyl)–4–(2–chlorophenyl)–1,4–dihydro–6–methyl pyridine–3,5–dicarboxylate monobenzene sulphonate (Structure III).

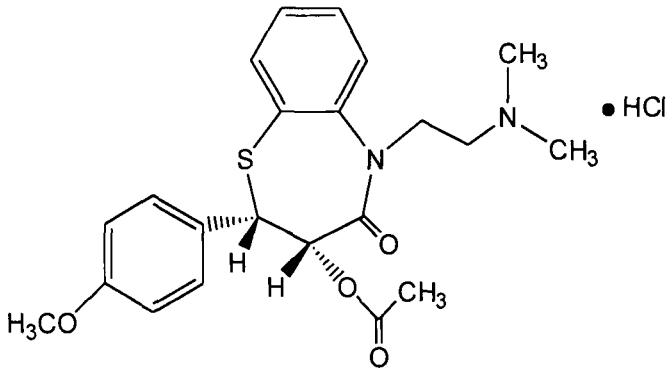


Structure III

It also belongs to dihydropyridine group of calcium channel blockers, undergoing photodegradation and showing almost similar therapeutic behaviour.

Diltiazem hydrochloride ($C_{22}H_{26}N_2O_4S$. HCl)

Diltiazem hydrochloride has been defined in the pharmacopoeias as (+) —5 —[2 — (dimethylamino)ethyl] — cis —2 , 3 — dihydro — 3 — hydroxy — 2 — (p — methoxyphenyl) — 1,5 — benzothiazepin — 4 (5H) — one acetate, containing not less than 98.5% and not more than 101.5% of $C_{22}H_{26}N_2O_4S$. HCl (Structure IV), calculated on the dried basis.



Structure IV

Diltiazem bears the peripheral and coronary vasodilation therapeutic properties. It lowers blood pressure and has some effect on cardiac conduction. It is orally administered for the management of angina pectoris and hypertension, and may also be given intravenously in the treatment of atrial fibrillation or flutter and paroxysmal supraventricular tachycardia.

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Chapter - 2

Spectrophotometric method for the determination of nifedipine with 4-(methylamino)phenol and potassium dichromate

Introduction

Nifedipine, chemically dimethyl–1,4–dihydro–2,6–dimethyl–4–(2–nitrophenyl) pyridine–3,5–dicarboxylate and pharmacologically a selective L–type slow calcium channel antagonist [1–2], is official in United States Pharmacopoeia XXIII and British Pharmacopoeia [3–4]. It is commonly used as an antihypertensive and potent arterial vasodilator in the management of angina and various other cardiovascular disorders [5]. It is also used as a probe drug to assess cytochrome P–450 III A4 enzyme activity *in vivo* [6]. Nifedipine decreases cyclic guanosine monophosphate in hypoxic lungs like inhaled nitric oxide, exhibits dose dependent depressive effect and causes some common side effects due to excessive vasodilation [7–9].

Several HPLC [6,10–12], reversed phase HPLC [13–14], HPTLC [15], GC [12,16–19] and voltammetric [20] methods have been reported for the assay of nifedipine and its related compounds in pharmaceuticals. A variety of HPLC and GC methods are now widely used for the determination of nifedipine concentration in biological fluids because of their sensitivity and specificity. These methods have adequate sensitivity to assay lower concentrations of the drug and hence use of these methods is justified when the sample matrix is complex and nifedipine concentration is low as in the case with the biological samples. However, the sample matrix is usually less complex and analyte concentration levels are high in case of pharmaceuticals, hence it is required to develop fast, simple and inexpensive method that can be adopted for routine analysis. Therefore, spectrophotometry is still considered as a convenient and low cost technique.

Nifedipine was assayed [21] in pharmaceutical formulations based on the reaction with 4–dimethylaminobenzaldehyde and subsequent determination at 310 nm. Beer–Lambert's law was obeyed over the concentration range 5 – 60 μg mL^{-1}. Another spectrophotometric method has also been recommended for its determination involving the formation of blue complex with Folin–Ciocalteau reagent [22]. A kinetic spectrophotometric method was described for its determination in dosage forms. The method was based on its oxidation by potassium permanganate at neutral pH [23]. In commercial dosage forms, UV–spectrophotometry has also been utilised for its estimation [24–25]. Nifedipine has also been determined using 3,4,5–trimethoxybenzaldehyde [26]. The Beer's law was obeyed over the concentration range 10 – 70 μg mL^{-1} with RSD of 1.5%. The quantification of nifedipine in combined dosage forms were made using first derivative [12] and second derivative [27] spectra of their solutions in methanol and 0.1 N HCl, respectively.

This chapter describes a new method for the determination of nifedipine in commercial dosage forms. The method depends on the reduction of nitro group to hydroxylamino group, which then reacted with N–methyl–1,4–benzoquinoneimine to form coloured product. The reduction of nitro group of nifedipine to hydroxylamino derivative was studied with respect to heating time and concentration of Zn / NH_4Cl. The effects of reagent's concentration, buffer and time on the formation of chromophore were investigated.

Experimental

Apparatus

Spectral runs and absorbances were recorded on a Spectronic 20D+ spectrophotometer (Milton Roy, USA). pH–meter model L1–10T (Elico, India) was used to measure the pH.

Materials and Reagents

(i) 0.1% ethanolic solution of reduced nifedipine was prepared by heating a mixture of 100 mg pure nifedipine (J.B. Chemicals and Pharmaceuticals Ltd., India) dissolved in 20 mL ethanol, 15 mL of 10% aqueous solution of ammonium chloride (Loba Chemie, India) and 2 g of zinc dust for 8 minutes on a water-bath at 100±1 °C. The content was cooled at room temperature and diluted by adding 30 mL of ethanol. It was filtered on whatmann no. 42 filter paper and washed with ethanol. The filtrate and washings were diluted to volume in a 100 mL volumetric flask. The whole experiment was performed in dark and in amber coloured glasswares. This solution was stable at room temperature for about 4 days.

(ii) A buffer solution was prepared by mixing appropriate volumes of 1 M hydrochloric acid (E. Merck, India) and 1 M sodium acetate trihydrate (Ranbaxy Chemicals, India).

(iii) 0.2% aqueous solution of 4–(methylamino)phenol (Loba Chemie) was prepared in doubly distilled water. It was always freshly prepared after every five hours.

(iv) 0.01 M potassium dichromate (Loba Chemie) was prepared by dissolving 0.7355 g in doubly distilled water and made up to 250 mL.

Recommended Procedure

Aliquots of 0.05 –1.75 mL of reduced nifedipine (1 mg mL^{-1}) were transferred into a series of 10 mL standard volumetric flasks and then 2.5 mL of buffer solution (pH = 2.9), 1.45 mL of 0.2% 4–(methylamino) phenol and 1.2 mL of 0.01 M potassium dichromate were added to each flask successively. The solutions were allowed to stand at room temperature for 18 minutes and then made upto the mark with doubly distilled water. The absorbance values of the

final coloured solutions were measured at 525 nm against a reagent blank. The amount of the drug was computed from a Beer–Lambert's plot.

Analysis of pharmaceutical formulations

10 tablets of nifedipine (each claiming 10 mg) were finely powdered and thoroughly mixed. The powdered mixture was transferred in a conical flask. 20 mL of ethanol was added and gently shaken for 2 – 3 minutes. Then 15 mL of 10% ammonium chloride solution and 2 g of zinc dust were added and heated on a water bath for 8 minutes. After cooling at room temperature, the mixture was added with 30 mL of ethanol and filtered on whatmann filter paper no. 42 in a 100 mL standard volumetric flask. The residue was washed with enough ethanol and finally made upto the mark. Nifedipine content was determined using the recommended procedure.

Investigation of stability

The stability of nifedipine under the experimental conditions was investigated by incubating 120 μg mL^{-1} in distilled water or ethanol containing common excipients at 30 °C for 3 hours in the absence of all lights. At regular time interval the concentration of nifedipine was determined by the proposed and reference methods [23].

Results and discussion

The nitro compounds undergo reduction by catalytic hydrogenation in the presence of metals (Zn, Fe, Sn) and other suitable reagents like, HCl, NH_4Cl, NaOH or KOH. Under the proposed condition, nifedipine is reduced to hydroxylamino derivative by Zn / NH_4Cl.

The primary aromatic amines react with 4–(methylamino)phenol and an oxidising agent such as dichromate [28], N–bromosuccinimide [29], peroxydisulphate [30] or iodylbenzoate [31] to form a purple red product. It is believed that on oxidation 4–(methylamino)phenol produces N–methyl–1,4–benzoquinoneimine. The primary aromatic amines react with N–methyl–1,4–benzoquinoneimine to form chromophore, which absorbed maximally at 520 – 530 nm. In this study the reaction of hydroxylamino derivative of nifedipine with 4–(methylamino)phenol and potassium dichromate may be assumed to proceed in an analogous manner as the product absorbed maximally at 525 nm (Fig. 2.1) [28]. The stoichiometric ratio of hydroxylamino derivative to N–methyl–1,4–benzoquinoneimine was determined by Job's method [32] and was found to be 2 : 1 (Fig. 2.2). The chromophore formed was found to be positively charged at pH 2.9 as it was adsorbed on cation–exchange resin beads. The possible reaction sequence is presented in Scheme 2.1.

The optimum conditions for the reduction of nifedipine to hydroxylamino derivative were established via a number of preliminary experiments. The effect of variables on the reduction of the drug was studied by taking a separate 10 mL aliquots of 0.1% nifedipine solution.

Effect of heating time

The aliquot of nifedipine was mixed with 200 mg of zinc dust and 1.5 mL of 10% ammonium chloride solution and the content was heated on a water bath at 100±1 °C. One mL aliquot of this solution was subjected to colour development. The results showed that the intensity of the colour was reached to its maximum at 7 minutes of heating (Fig. 2.3) and remained unchanged even after 10 minutes. Therefore, a heating time of 8 minutes was recommended for reduction.

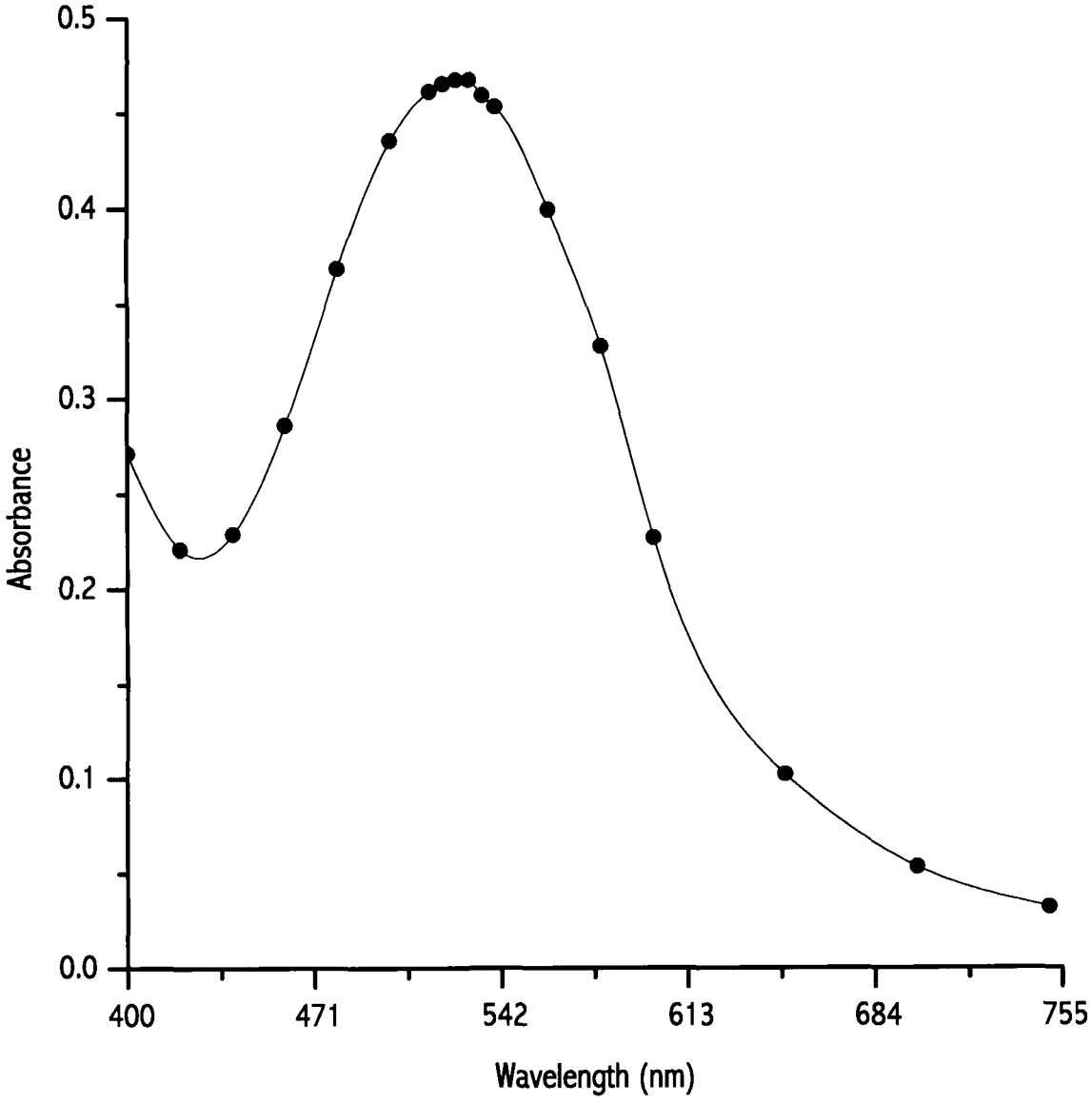


Fig. 2.1. Absorption spectrum of the hydroxylamino derivative of nifedipine and N– methyl–1,4–benzoquinoneimine complex.

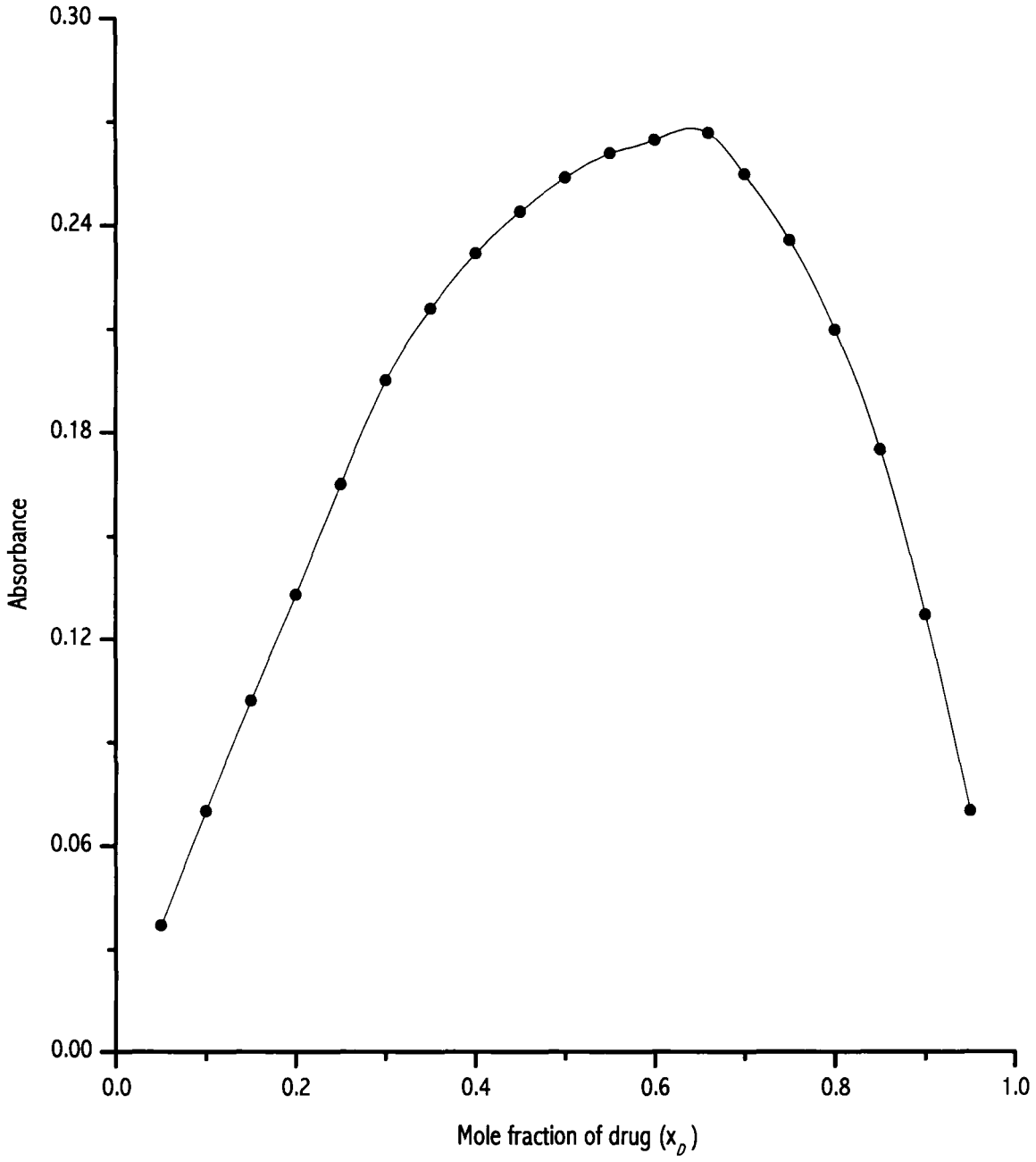
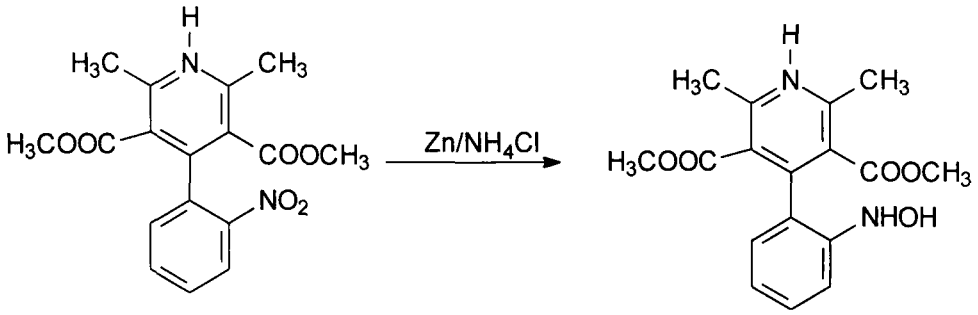
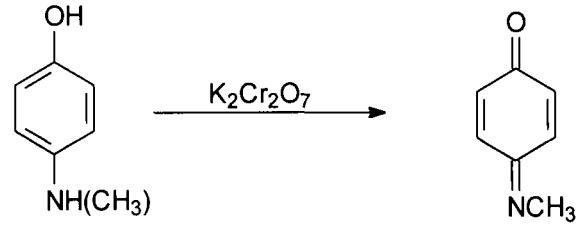


Fig. 2.2. Job's plot for the hydroxylamino derivative of nifedipine and N–methyl–1, 4–benzoquinoneimine (each 2.89 x10^{-3} M).

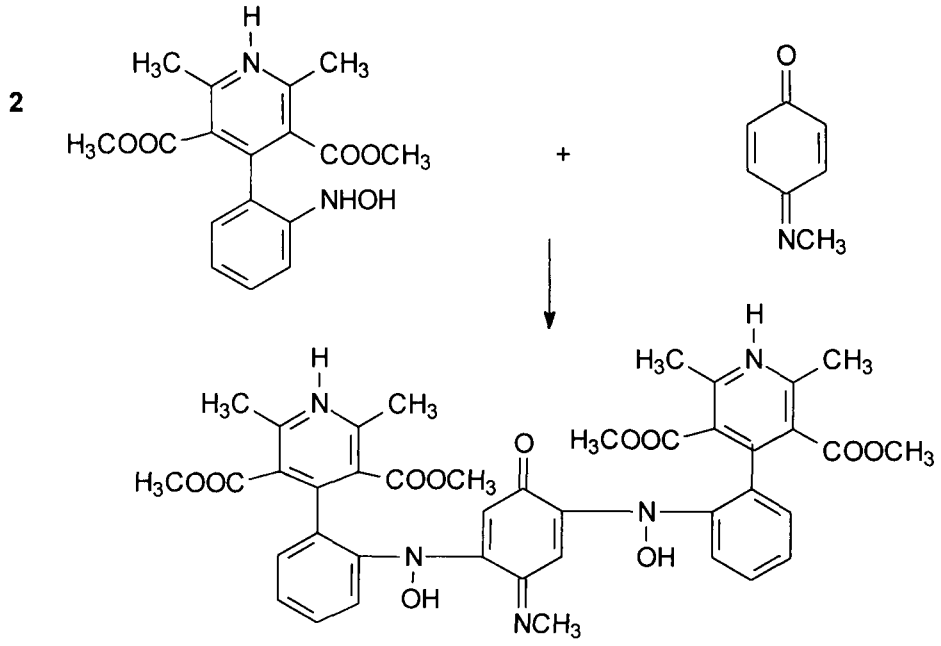
(a)Reduction of nifedipine to hydroxylamino derivative



(b)Formation of N-methyl-1,4-benzoquinoneimine



(c) Coupling with hydroxylamino derivative of drug



Scheme 2.1

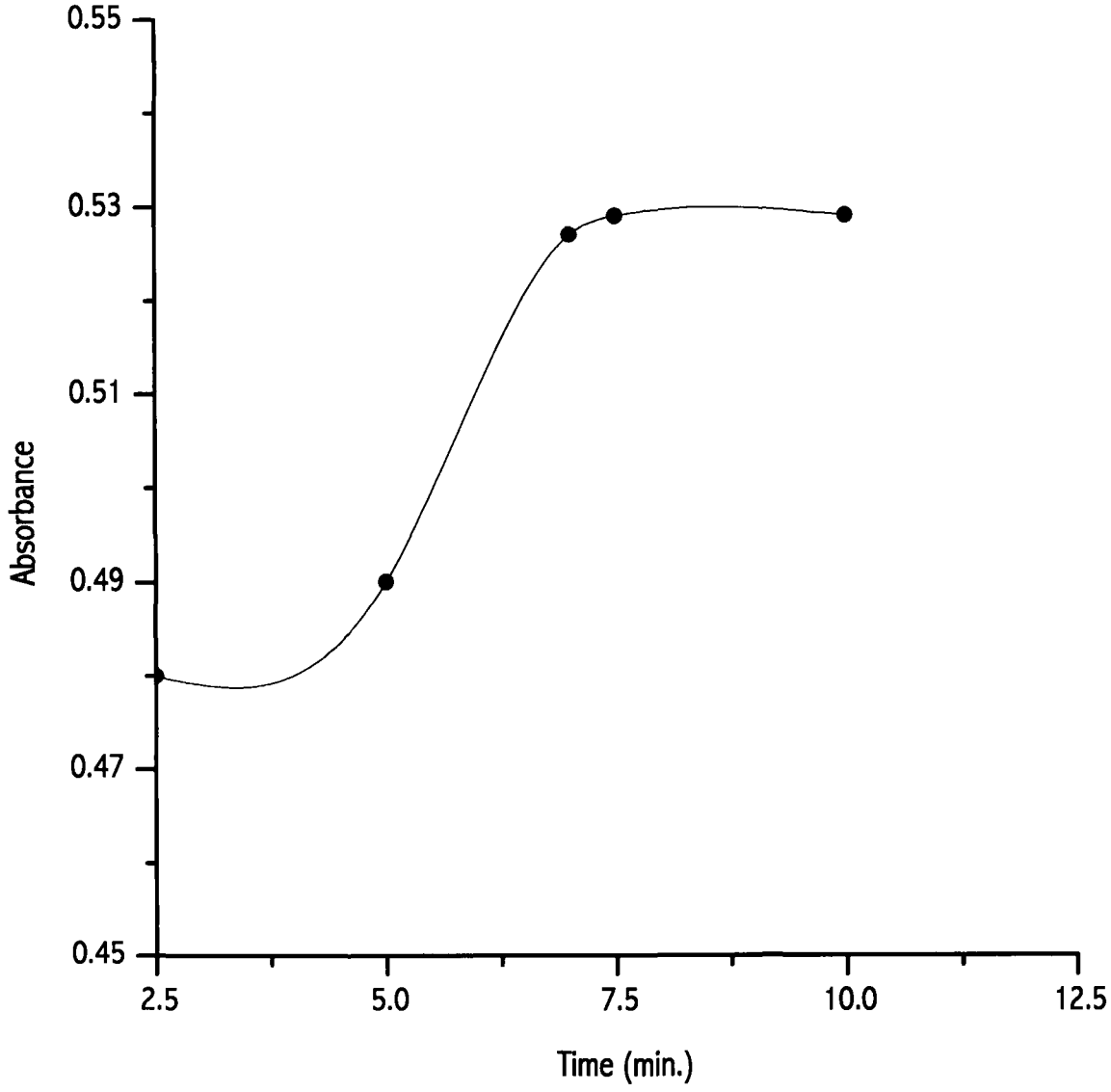


Fig. 2.3. Effect of heating time on the reduction of nifedipine.

Effect of Zinc dust

The effect of the amount of zinc dust on the reduction of 10 mg nifedipine in the presence of 1.5 mL of 10% ammonium chloride solution was studied. It was observed that the absorbance of the coloured solution increased upto 150 mg of zinc dust and then remained constant at higher amounts (Fig. 2.4). Hence, 200 mg of zinc dust was taken as optimum value for reduction.

Effect of ammonium chloride solution

To study the effect of the concentration of ammonium chloride solution on the reduction of nifedipine, 10 mL of 0.1% nifedipine was mixed with 200 mg of zinc dust and varying volumes of 10% ammonium chloride solution. The content was heated on a water bath at 100±1 °C for 8 minutes. A plot of absorbance vs. volume of ammonium chloride solution showed that the highest absorbance was obtained with 1.25 mL and remained constant beyond this volume (Fig. 2.5). Therefore, 1.5 mL of 10% ammonium chloride solution was taken to reduce the drug for further studies.

The optimum conditions for the development of the proposed method were established by varying the parameters one at a time and observing the effects produced.

Effect of buffer solution

Fig. 2.6 shows the effect of HCl–CH_3COONa buffer on the formation of chromophore. A constant absorbance was observed in the pH range of 2.75 – 3.1. In this study, therefore, 2.5 mL of pH 2.9 buffer solution was used throughout the experimental investigations.

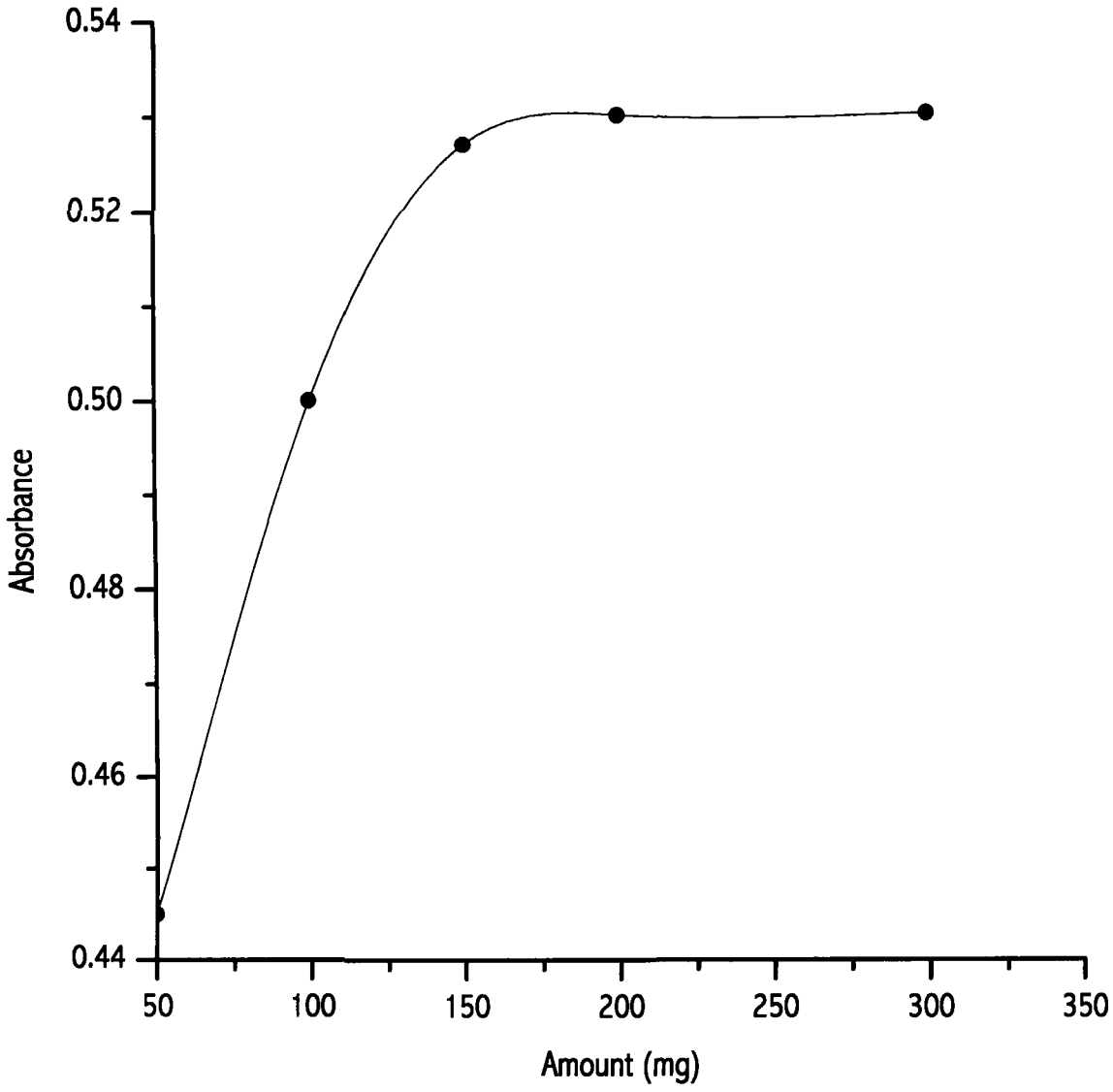


Fig. 2.4. Effect of zinc dust on the reduction of nifedipine.

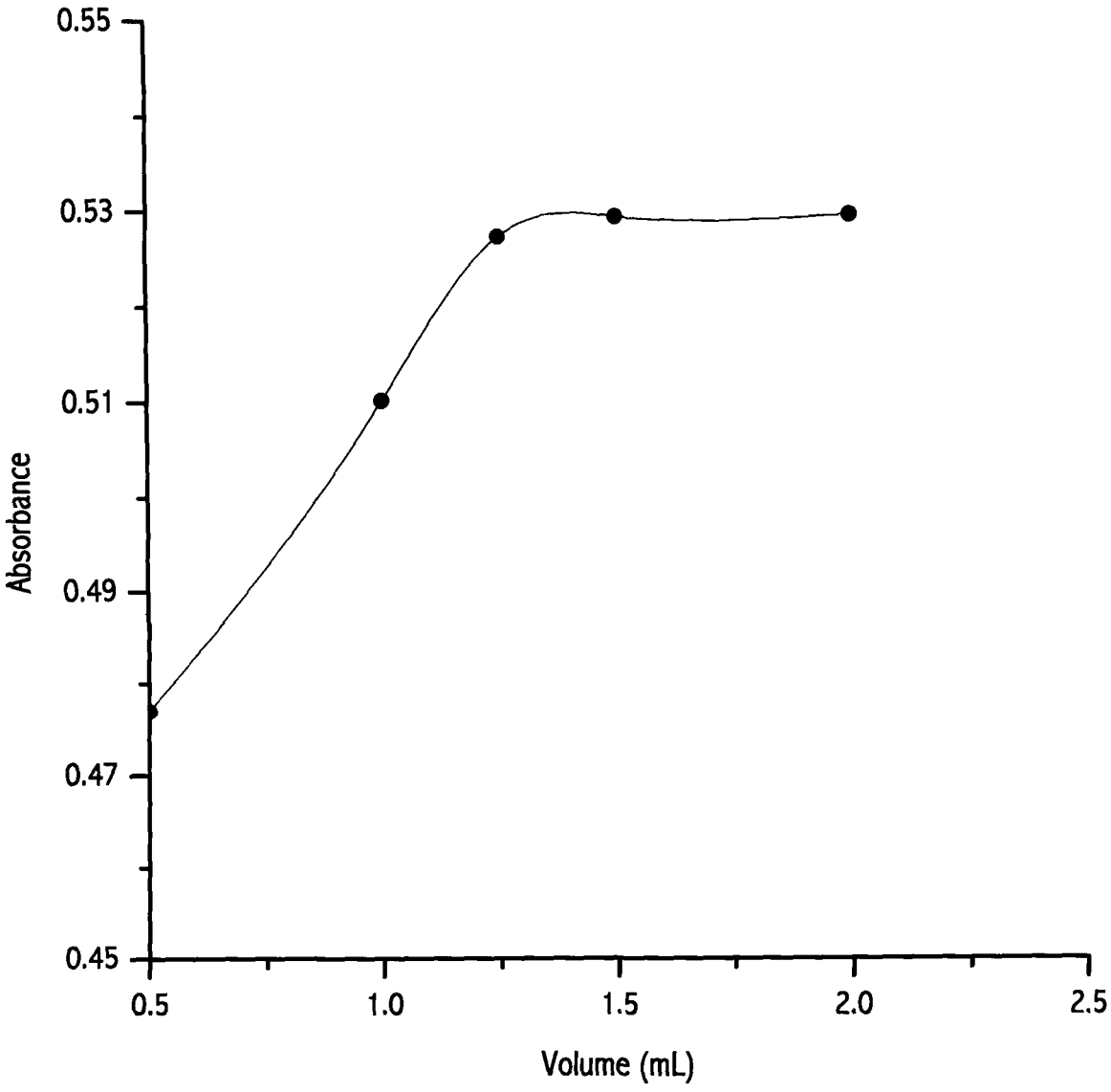


Fig. 2.5. Effect of 10% aqueous ammonium chloride solution on the reduction of nifedipine.

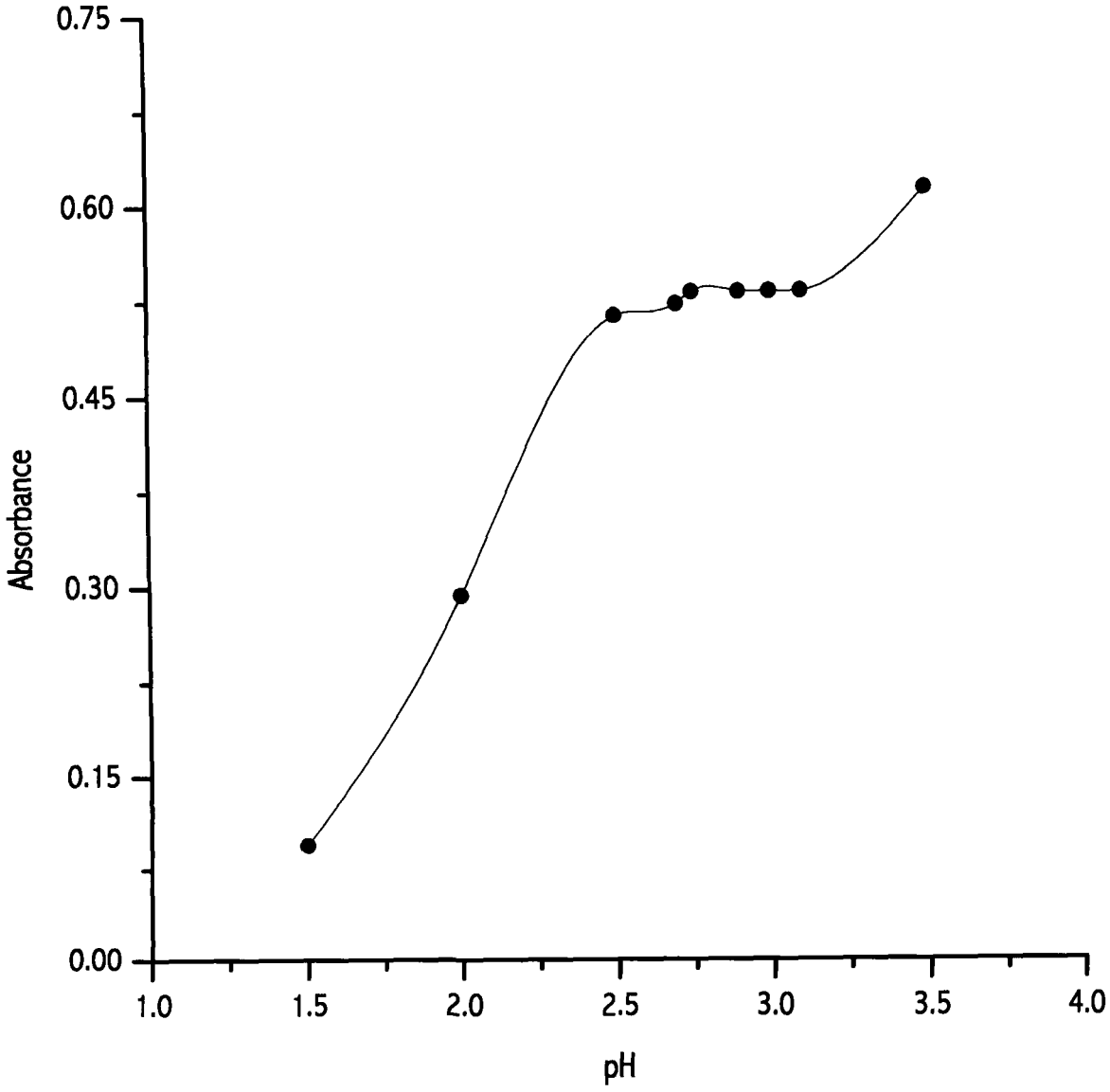


Fig. 2.6. Influence of the pH of HCl–CH_3COONa buffer solution on the formation of chromophore.

Effect of time

To 1 mL of 0.1% reduced drug, 2.5 mL of buffer solution (pH = 2.9), 1.45 mL of 0.2% 4–(methylamino)phenol solution and 1.2 mL of 0.01 M potassium dichromate were added and kept at room temperature (25 °C) for colour development. The coloured product was diluted to 10 mL with distilled water and the absorbance was measured at 525 nm against a reagent blank after 18 minutes. The intensity of the colour was reached to maximum after 15 minutes (Fig. 2.7) and remained constant for one hour.

Effect of 4–(methylamino)phenol solution

1 mL of 0.1% reduced drug, was mixed with 2.5 mL of buffer solution (pH = 2.9), varying volumes of 0.2% 4–(methylamino)phenol and 1.2 mL of 0.01 M potassium dichromate and the contents were allowed to stand at room temperature for 18 minutes. The results showed that a constant absorbance was found in the range of 1.4 – 1.5 mL (Fig. 2.8). Therefore 1.45 mL of 0.2% 4–(methylamino)phenol was used in all the subsequent works.

Effect of potassium dichromate solution

In order to study the effect of potassium dichromate concentration, the reaction was carried out in a series of 10 mL volumetric flasks containing 100 μg mL^{-1} reduced drug, 2.5 mL of buffer solution (pH = 2.9), 1.45 mL of 0.2% 4–(methylamino)phenol solution. This was followed by different volumes of 0.01 M potassium dichromate ranging from 0.3 to 1.5 mL. The results (Fig. 2.9) indicate that the highest intensity and reproducible results are obtained on using 1 mL of 0.01 M potassium dichromate. Therefore, 1.2 mL of this reagent was used throughout this work.

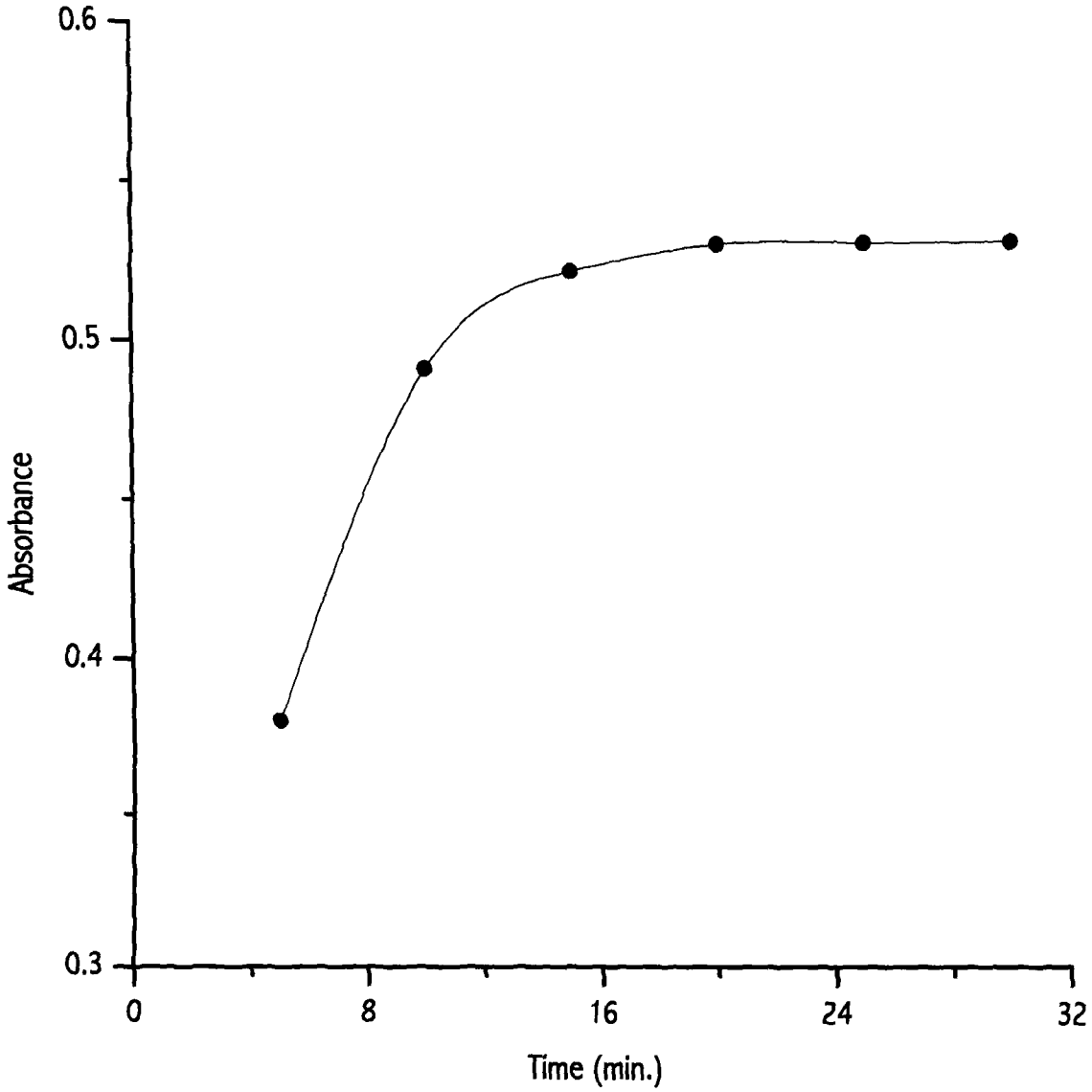


Fig. 2.7. Effect of time on the formation of chromophore of hydroxylamino derivative of nifedipine and N–methyl–1,4–benzoquinoneimine.

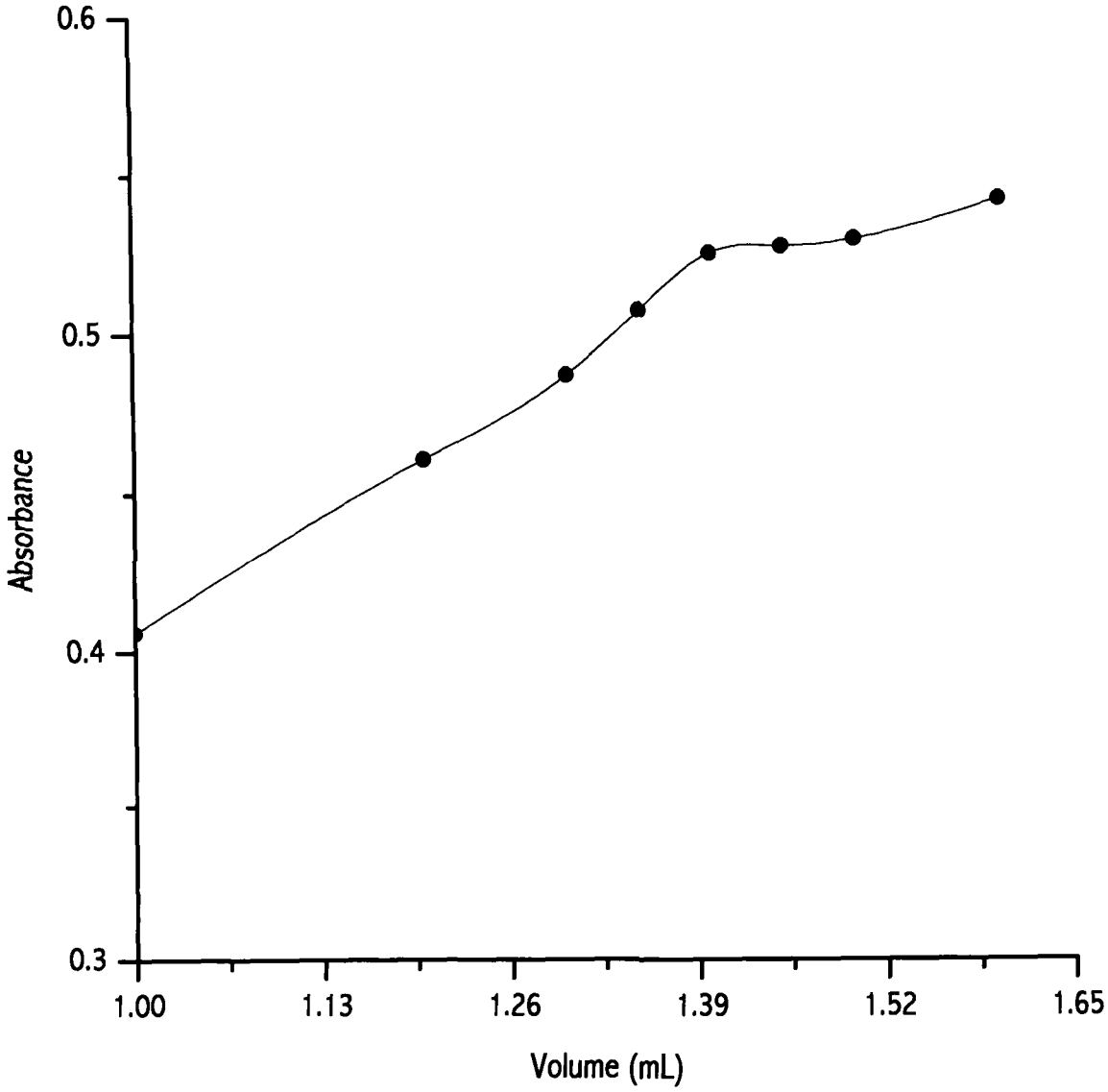


Fig. 2.8. Effect of 0.1% aqueous solution of 4–(methylamino)phenol on the chromophore formation.

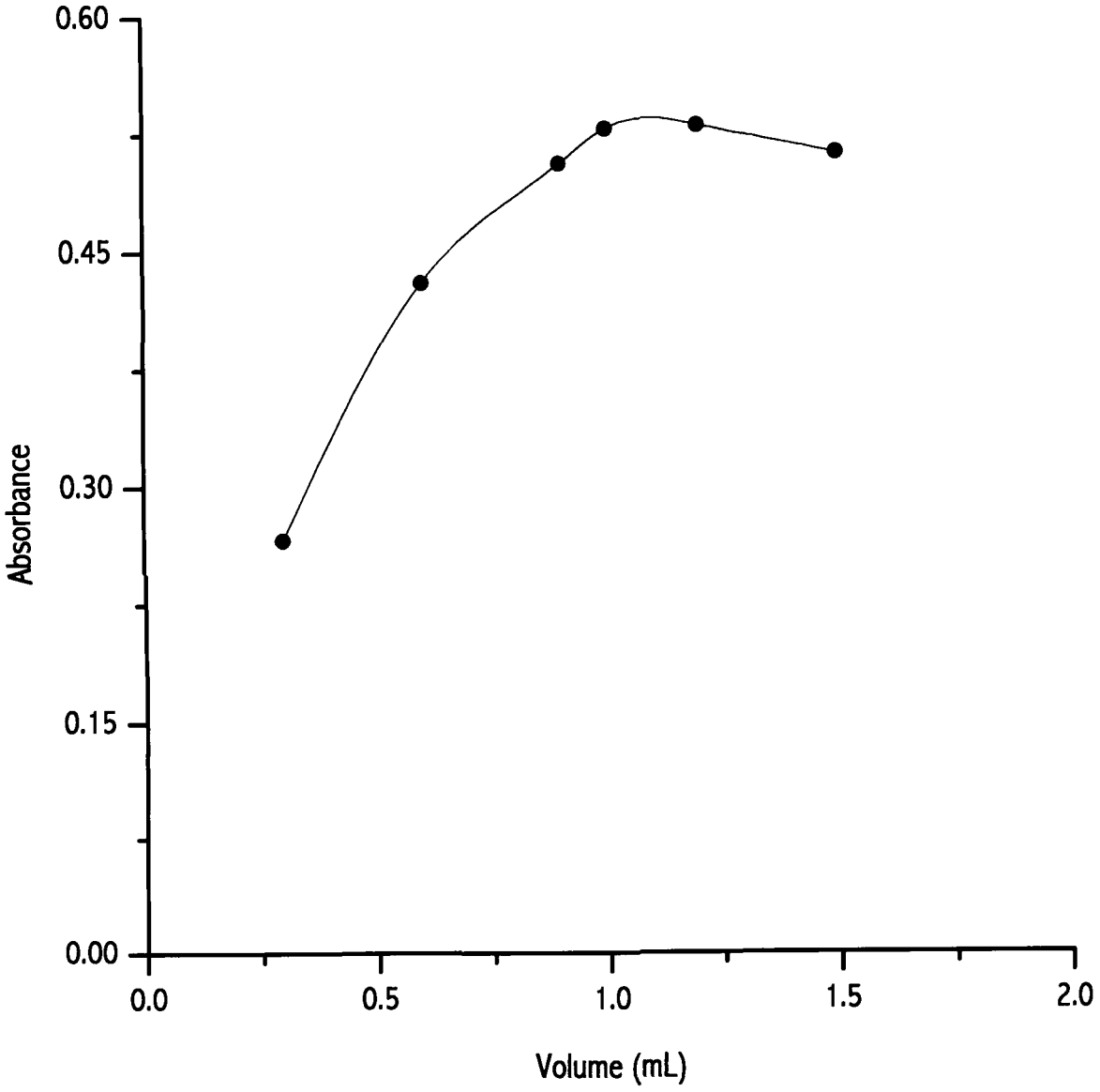


Fig. 2.9. Effect of 0.01 M potassium dichromate solution on the formation of chromophore.

Analytical data

Under the optimum experimental conditions, a calibration curve was constructed by plotting absorbance at 525 nm versus concentration (Fig. 2.10). Beer's law was obeyed within a concentration range of 5 – 175 μg mL^{-1}. Regression analysis using the method of least square was made to evaluate the slope, intercept and correlation coefficient. The linear regression equation and correlation coefficient are $A = 1.3 \times 10^{-3} + 5.3 \times 10^{-3} C$ (A, absorbance at 525 nm; C, concentration in μg mL^{-1}) and $r = 0.9999$ which indicates an excellent linearity. The molar absorptivity, detection limit and variance [33], standard deviations of intercept and slope [34] were found to be 1.9×10^3 L mol^{-1} cm^{-1}, 1.1 μg mL^{-1} and 1.2×10^{-5}, 1.9×10^{-3} and 1.6×10^{-5}, respectively. The small value of variance marks the negligible scattering of the experimental data points from the line of regression.

The error, S_c, in the determination of a given concentration of nifedipine was calculated by statistical analysis of the calibration data using the relation [35].

$$S_c = \frac{S_o}{b}\left(1 + \frac{1}{n} + \frac{(A - A')^2}{b^2(\Sigma C^2 - nC'^2)}\right)^{1/2}$$

where C' and A' = average concentration and average absorbance values, respectively, for n standard solutions. Fig. 2.11 shows the graph of S_c against the concentration of nifedipine. The value of S_c reached to minimum when the actual absorbance was equal to the average absorbance in the calibration graph. Thus the minimum error was found in the determination of about 100 μg mL^{-1} nifedipine. The value of S_c also allows to establish the confidence limits at the selected level of significance [35]. The results are shown in the Fig 2.12 in the form of percent uncertainty on the concentration at 95% confidence level. It is apparent from the figure

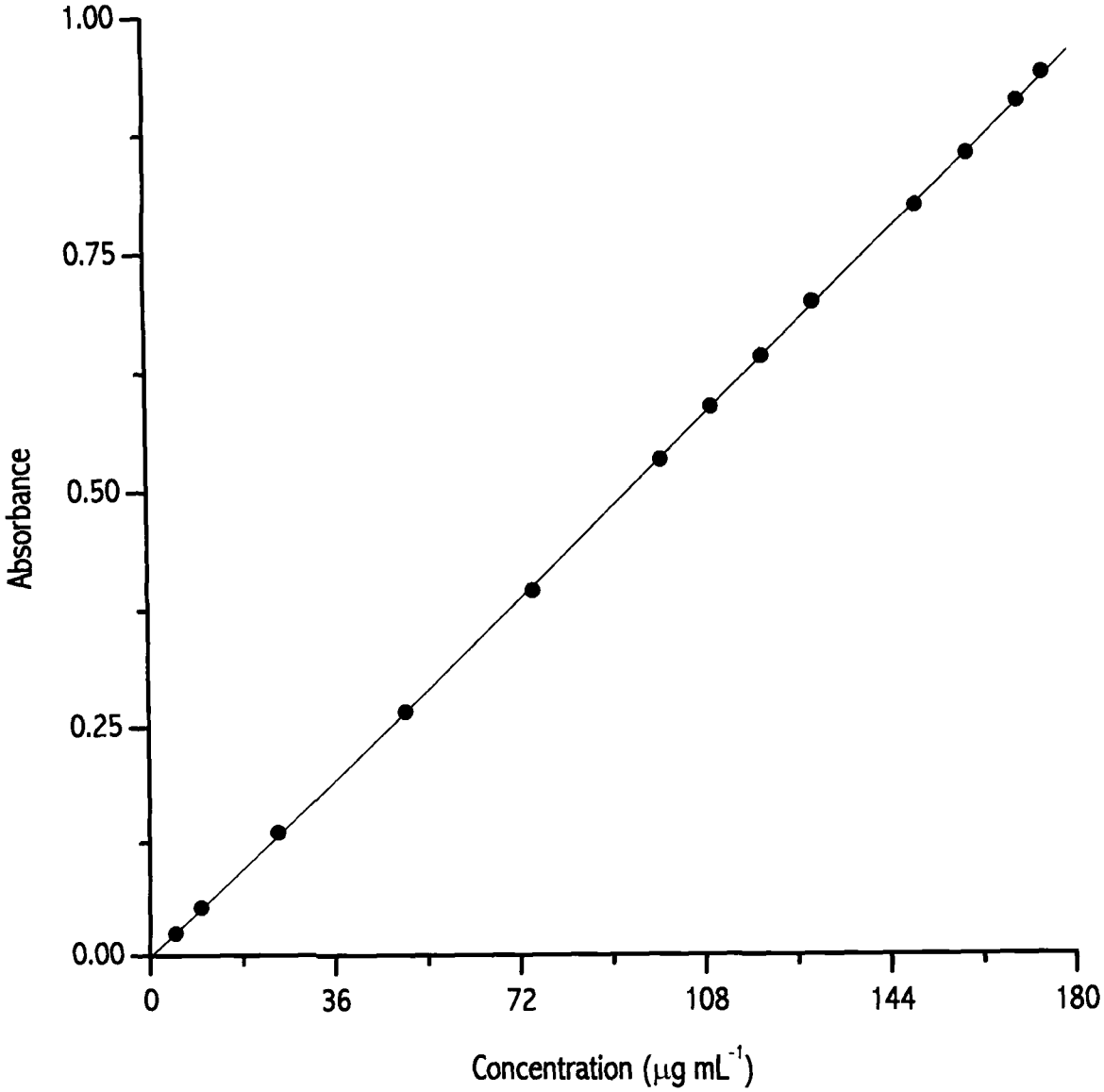


Fig. 2.10. Calibration curve for the determination of nifedipine.

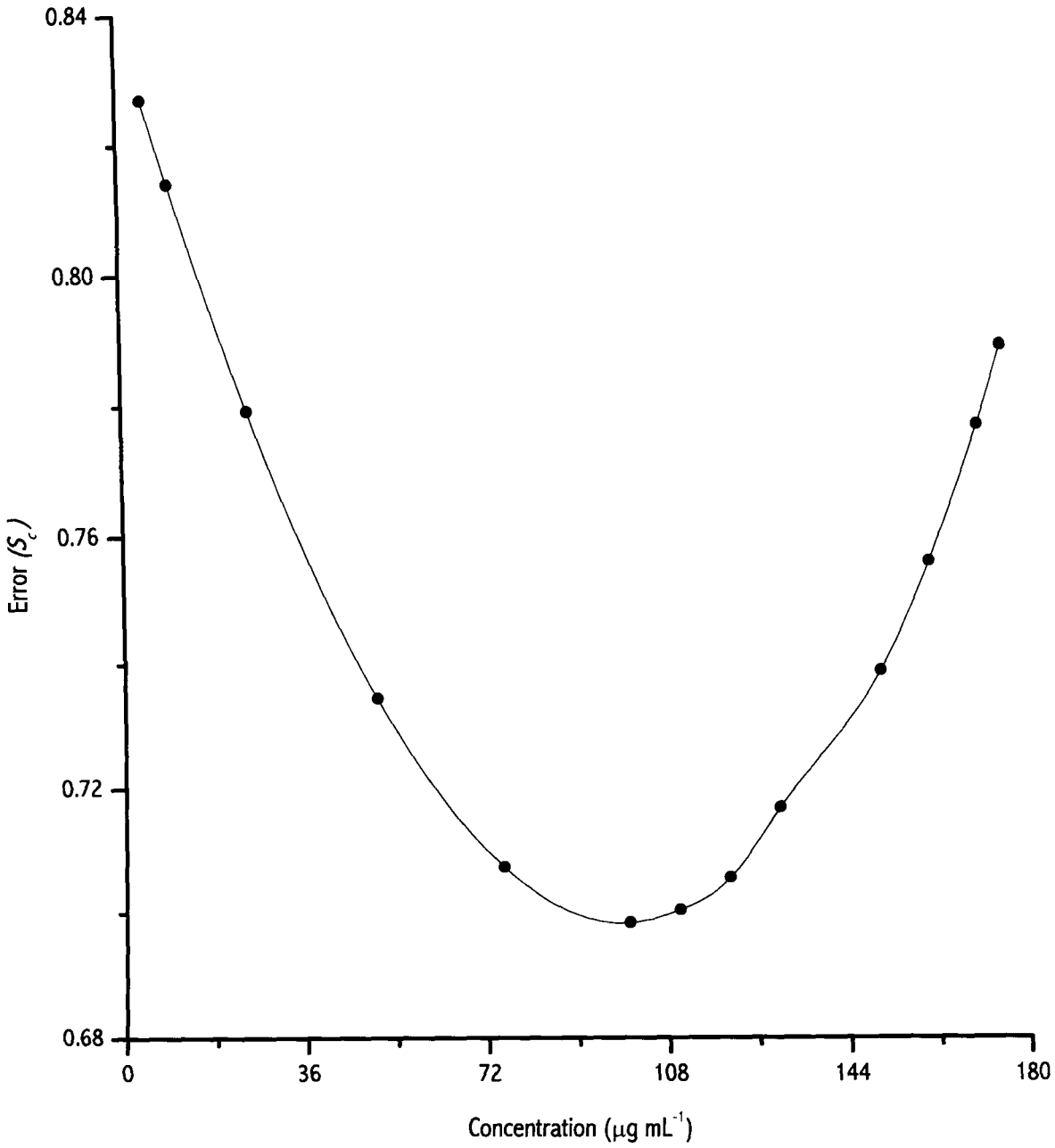


Fig. 2.11. Error (S_c) in the determination of the concentration of nifedipine.

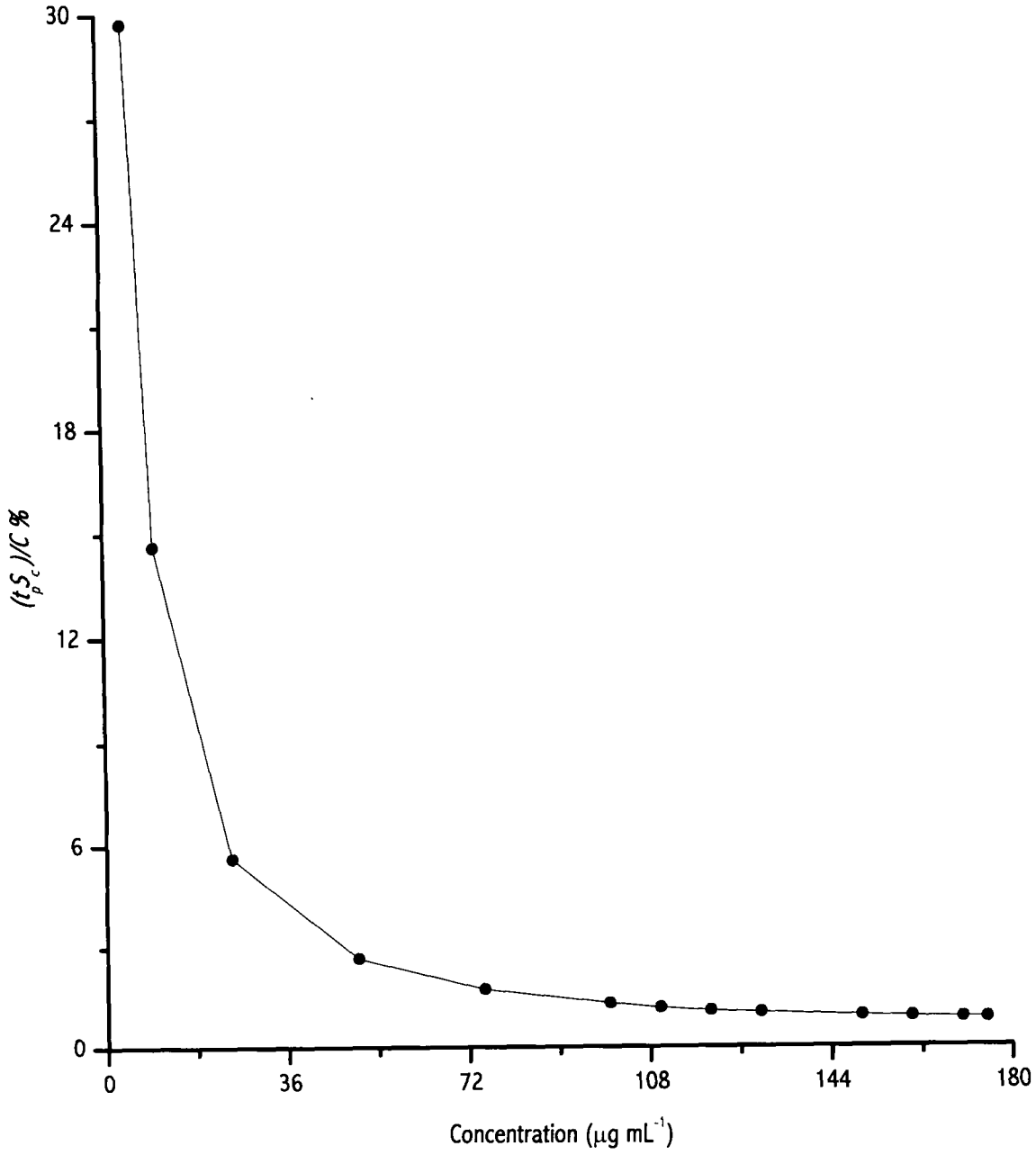


Fig. 2.12. Variation of confidence limit at 95% confidence level in the form of percent uncertainty on the concentration.

that the relative uncertainty on the concentration can be calculated directly over the full range of the concentration tested and hence, confidence limit can be established.

The stability experiment conducted in the presence of commonly encountered excipients such as starch, talc, lactose and magnesium stearate revealed the fact that under the conditions, no degradation of nifedipine was detected. The drug and its photodegradation product, 4–(2–nitrosophenyl)–pyridine homologue may undergo reduction with Zn/ NH_4Cl to yield hydroxylamino derivative. However, the determination was done under a condition where contact with light was completely avoided.

The reproducibility of the method was checked by ten replicate determinations at the concentration levels of 60, 100, 120 and 150 μg mL^{-1}. The percent relative standard deviations were found to vary between 0.3 – 0.8.

The accuracy of the method was demonstrated by recovery experiments, which were carried out by adding a fixed amount of pure drug to the preanalysed formulations. The analytical results obtained from these investigations are summarised in Table 2.1, which indicates that the common additives and excipients did not interfere with the determination. The percent relative standard deviations can be considered to be very satisfactory.

The proposed method was compared favourably with other existing UV–visible spectrophotometric methods (Table 2.2). It is evident from the table that the method has advantages of wider linear dynamic range and high precision (%RSD = 0.3 – 0.8 and 0.4 – 0.7 for pure and dosage forms, respectively). Some commercial dosage forms were successfully analysed by the proposed method and official B.P. method [4]. The results (Table 2.3) were compared statistically by Student's *t*–test and variance ratio *F*–test, which indicates that there is no significant difference between the methods compared.

Table 2.1. Spectrophotometric determination of nifedipine in pharmaceutical formulations by standard addition method.

Preparations	Amount ($\mu g\ mL^{-1}$)			Recovery (%)	RSD (%)
	Taken	added	found		
Adalat Retard–10	30	30	60.1	100.1	0.8
	40	60	99.5	99.5	0.6
	80	40	120.4	100.3	0.5
Calcigard–10	30	30	59.5	99.2	1.0
	40	60	99.8	99.8	0.8
	80	40	120.7	100.6	0.5
Nicardia Retard–10	30	30	59.5	99.2	0.7
	40	60	100.4	100.4	0.4
	80	40	120.5	100.4	0.5

Table 2.2. Comparison of the proposed method with the existing spectrophotometric methods for the assay of nifedipine in pharmaceutical formulations.

Reagents	λ_{max} (nm)	Linear Dynamic Range ($\mu g\ mL^{-1}$)	Recovery (%)	RSD (%)	References
Potassium permanganate	530	18 – 44	99.5 – 101.3	1.5	[23]
3,4,5–trimethoxybenzaldehyde	365	10 – 70	100.2 – 102.9	1.5	[26]
4–dimethylaminobezaldehyde	310	5 – 60	97.8 – 98.5	–	[21]
* Extractive U.V.	237	0 – 10	97.8 – 98.9	–	[25]
Ethanol and phosphate buffer saline	340	–	99.7 – 99.9	–	[24]
Derivative U.V.	400	4 – 12	98.5 – 101.3	1.4	[12]
4–N–methylaminophenol and dichromate	525	5 – 175	99.7 – 100.5	0.6	This work

* Extracted into chloroform and the solvent was evaporated to dryness. Finally, the residue was dissolved in distilled water.

Table 2.3. Spectrophotometric determination of nifedipine in pharmaceutical formulations by the proposed method and B.P. method [4].

Preparations	Nominal composition (mg)	Proposed method			Reference Method			*F*–value[c]
		Recovery[a] (%)	RSD[a] (%)	*t*–value[b]	Recovery[a] (%)	RSD[a] (%)	*t*– value[b]	
Adalat Retard-10	10	99.9	0.6	0.3923	100.1	0.4	1.3975	2.0306
Calcigard-10	10	100.2	0.7	0.6675	100.2	0.5	0.8856	2.2168
Nicardia Retard-10	10	99.9	0.4	0.520	100.4	0.5	1.9876	1.0952

[a] Five independent analyses.

[b] *t*–value at 95% confidence level is 2.132 [36].

[c] *t*– value at 95% confidence level is 6.39 [36].

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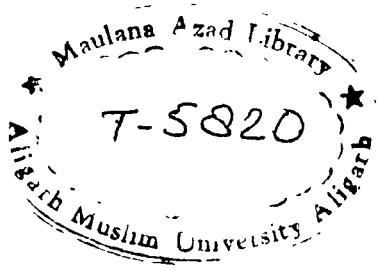

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Introduction

Verapamil hydrochloride, belonging to phenylalkylamine group of calcium channel blockers, is chemically 5–[N–(3,4–dimethoxyphenylethyl)methylamino]–2–(3,4–dimethoxyphenyl)–2–isopropyl valeronitrile hydrochloride [1]. The drug is officially listed in British, United States and Indian Pharmacopoeias [2–4]. It is a potent antihypertensive agent with significant depressant effects and has been recommended for intravenous therapy of supraventricular tachyarrhythmias [5].

The drug has been determined in biological fluids and for pharmaceutical dosage forms by a variety of analytical techniques such as high performance liquid chromatography [6–11], gas liquid chromatography [12], capillary gas chromatography [13], high performance thin layer chromatography [14], potentiometry [15] and potentiometry–conductometry [16]. The visible spectrophotometric methods are the instrumental methods of choice, which provides practical and significant economic advantages over the other methods. In the literature, only few spectrophotometric methods have been reported which are usually based on either extractable ion–pair complex formation with bromophenol blue, bromocresol purple, bromocresol green, bromothymol blue and methyl orange [17]; solochrome black–T, solochrome dark blue, solochrome cyanine R and fast sulfone FF [18]; erioglaucine and indigocarmine [19]; alizarin red–S [20] or charge transfer complex formation with polyhalo/polycyanoquinones [21–22] and azo dyes [23]. However, some of these methods suffer from one or other disadvantages such as low sensitivity, lack of selectivity and simplicity.

Chloramine–T is a strong oxidant in both acidic and alkaline media (E_{red} = 1.138 at pH 0.65 and 0.5 at pH 12) [24–25]. In acidic aqueous solution, chloramine–T is thought to exist

in a complex series of equilibria [26] which indicates that the probable oxidising species in acidified chloramine–T is hypochlorous acid.

Chloramine–T was initially introduced as a disinfectant and antiseptic but nowadays widely used as an oxidant for various organic functional groups [27]. It has been used as a reagent for the spectrophotometric determination of p-aminobenzenesulphonamides [28] and sulphamethoxazole, tetracycline hydrochloride, amidopyrine, nifurtimox and isoniazid [29], which involves the addition of excess chloramine–T and the determination of unreacted reagent. Chloramine–T is also utilised in the titrimetric determination where the end point is detected with either a visual indicator [28, 30–32] or potentiometrically [31].

This chapter describes the spectrophotometric determination of verapamil hydrochloride based on its oxidation with chloramine–T in acidic medium producing a yellow chromophore.

Experimental

Apparatus

Spectronic 20D$^+$ spectrophotometer (Milton Roy, USA) with matched glass cuvettes was used for spectral runs and absorbance measurements.

Reagents

A 0.4% verapamil hydrochloride (Sigma, USA) solution was prepared in AR–grade methanol (S.D. Fine Chem. Ltd., India) and was further diluted according to the need. 1% solution of chloramine–T (E. Merck, India) and 5 M hydrochloric acid (E. Merck) were prepared in doubly distilled water.

Recommended procedure

Into a series of 10 mL volumetric flasks 1 mL of varying concentrations of verapamil hydrochloride, upto 340 μg mL^{-1}, were pipetted out. To each flask 2.5 mL of 1% chloramine–T solution and 6 mL of 5 M HCl were added and diluted to the mark with doubly distilled water. The content was mixed well and kept at room temperature for 15 minutes. The absorbances were measured at 425 nm against the reagent blank prepared simultaneously omitting the drug. The amount of drug in each sample were calculated either from the calibration graph or regression equation.

Analysis of verapamil hydrochloride in pharmaceutical formulations

Ten tablets of verapamil hydrochloride, equivalent to 400 mg of pure drug, were grounded to fine powder. The whole mass was stirred in methanol and filtered through whatmann no. 42 filter paper into a 100 mL volumetric flask. The residue was washed well with methanol. The filtrate and washings were diluted to 100 mL volume. This solution was diluted according to the need and analysed by the recommended procedure.

Results and discussion

Chloramine–T is well known oxidant and its oxidative behaviour resembles to that of the hypohalites. Bishop and Jennings [33], Morris *et. al.* [34] and Higuchi and Hussain [35] have studied the equilibria involved in acidified chloramine–T solution and suggested the formation of hypochlorous acid. This reacts with verapamil hydrochloride to form the relevant oxidation products. It is also known that N–bromosuccinimide reacts with tertiary amine [36]. In such studies, a methyl or methylene group attached to nitrogen was required and $>N–CH_2$

linkage was cleaved preferentially giving yellow coloured products. In a similar fashion it is believed that verapamil is oxidised by hypochlorous acid in which >N–CH_2 bond is cleaved resulting in the formation of aldehyde and secondary amine. The oxidation product absorbs maximally at 425 nm (Fig. 3.1). Therefore, based on the literature background and our findings, the reaction mechanism was proposed and given in Scheme 3.1.

The optimum conditions for the assay of verapamil hydrochloride were established via a number of preliminary experiments.

Effect of time

To investigate the effect of time on the colour development, 1 mL of 0.2% verapamil hydrochloride was pipetted in a 10 mL volumetric flask, 2.5 mL of 1% chloramine–T and 6 mL of 5 M HCl were added and diluted to the mark with doubly distilled water. The absorbance was recorded as a function of time. The results showed (Fig. 3.2) that the absorbance became constant after 13 minutes and remained unchanged upto 20 minutes. Thus absorbance was measured within the stability period.

Effect of chloramine–T concentration

To 1 mL of 0.2% verapamil hydrochloride, varying volumes (0.5 – 3.0 mL) of 1% chloramine–T and 6 mL of 5 M HCl were added. The coloured product was diluted to 10 mL with doubly distilled water and absorbances were measured against the corresponding reagent blanks after 15 minutes. The results (Fig. 3.3) showed that the highest absorbance was obtained with 2.25 mL, which remained constant with higher amounts of chloramine–T. Thus, 2.5 mL of 1% chloramine–T was added for colour development.

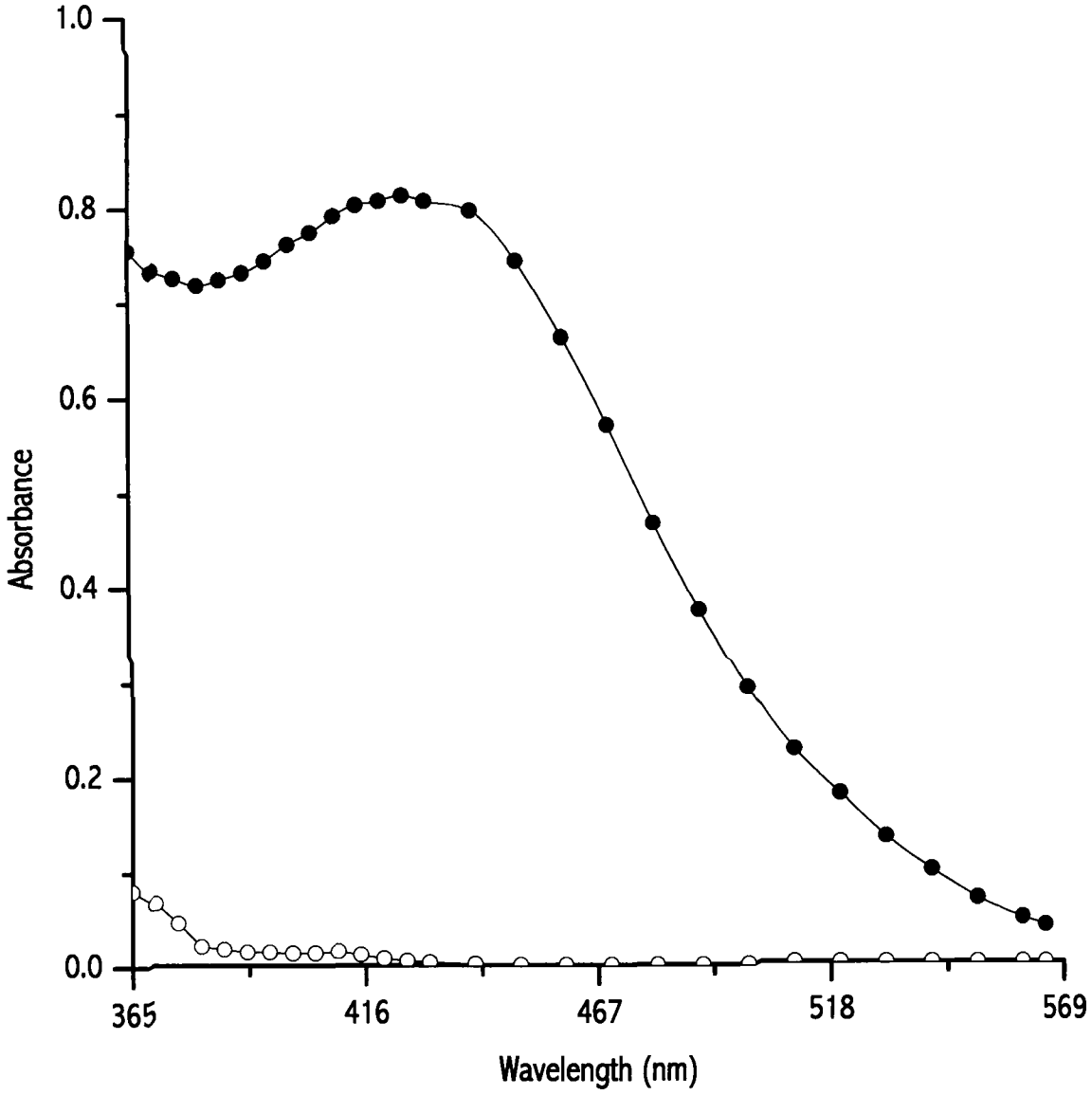
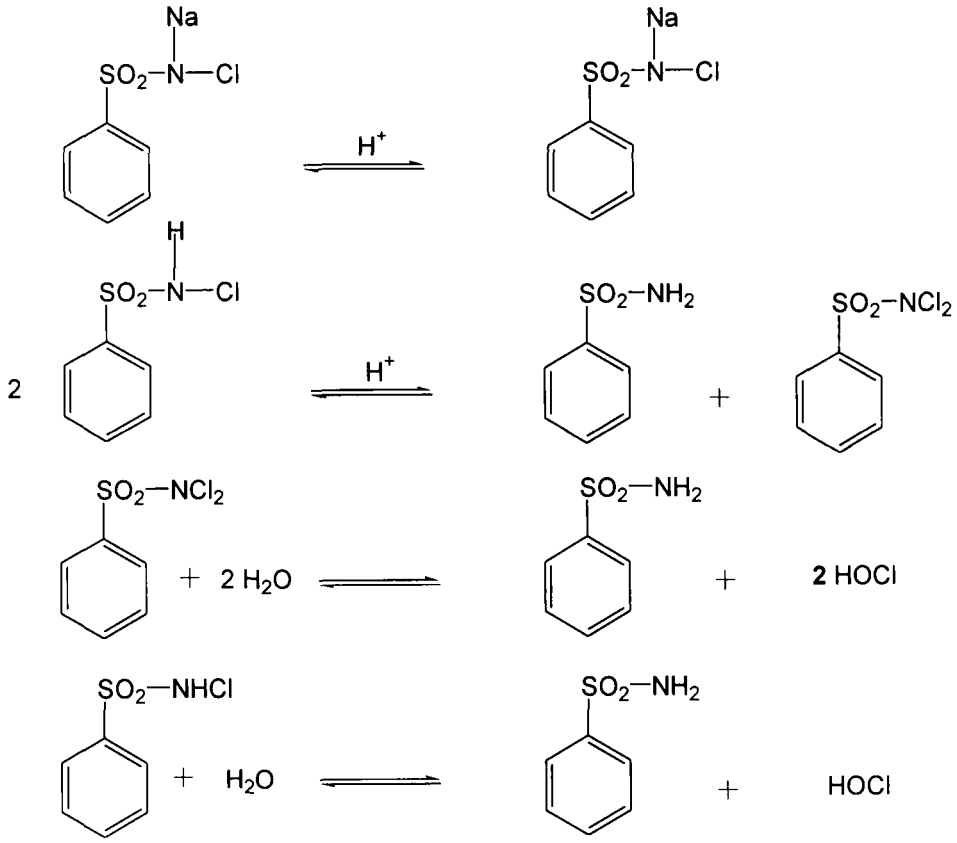
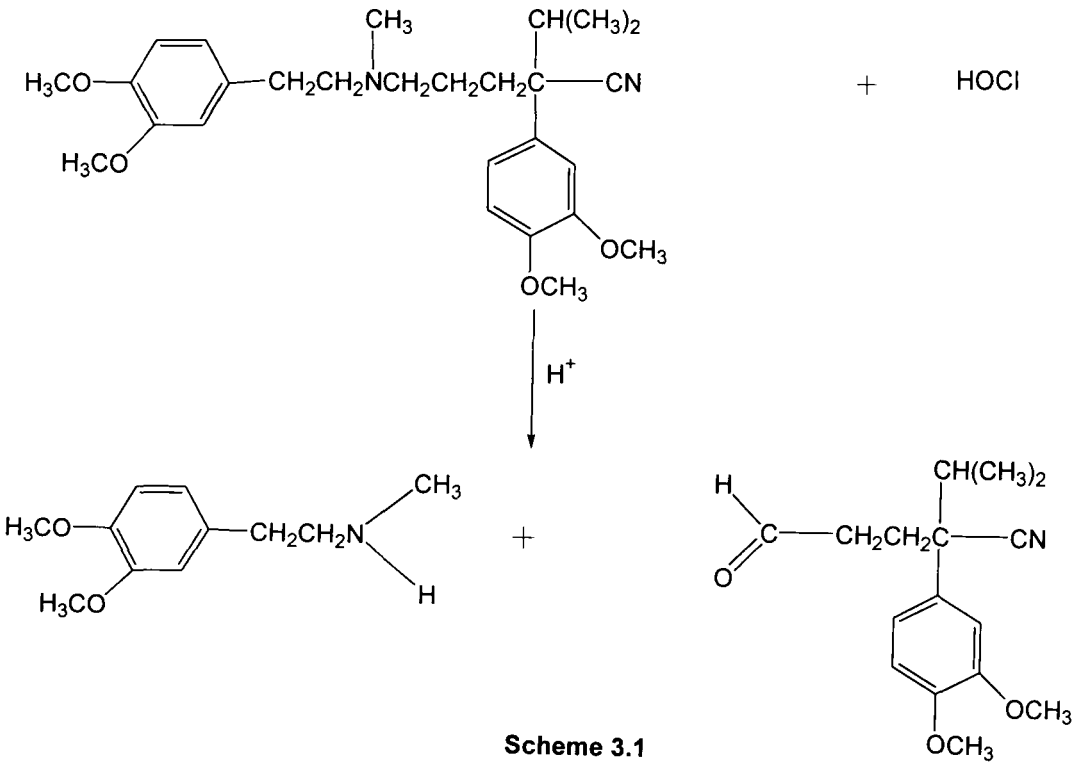


Fig. 3.1. Absorption spectra of the oxidation product of verapamil (•) and its reagent blank(o).

(1) Formation of hypochlorous acid



(2) Oxidation of verapamil by hypochlorous acid



Scheme 3.1

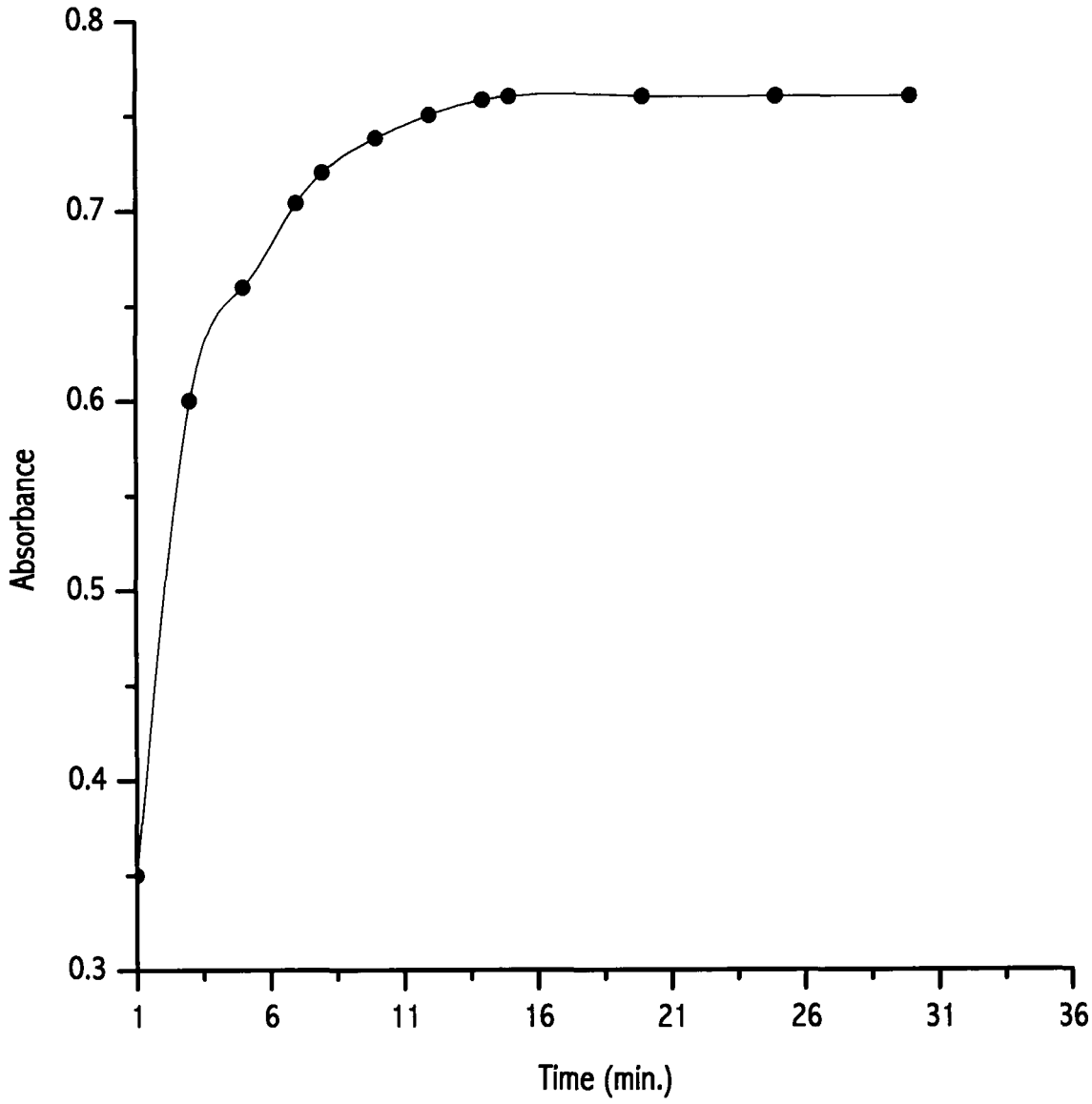


Fig. 3.2. Effect of time on the oxidation of verapamil hydrochloride.

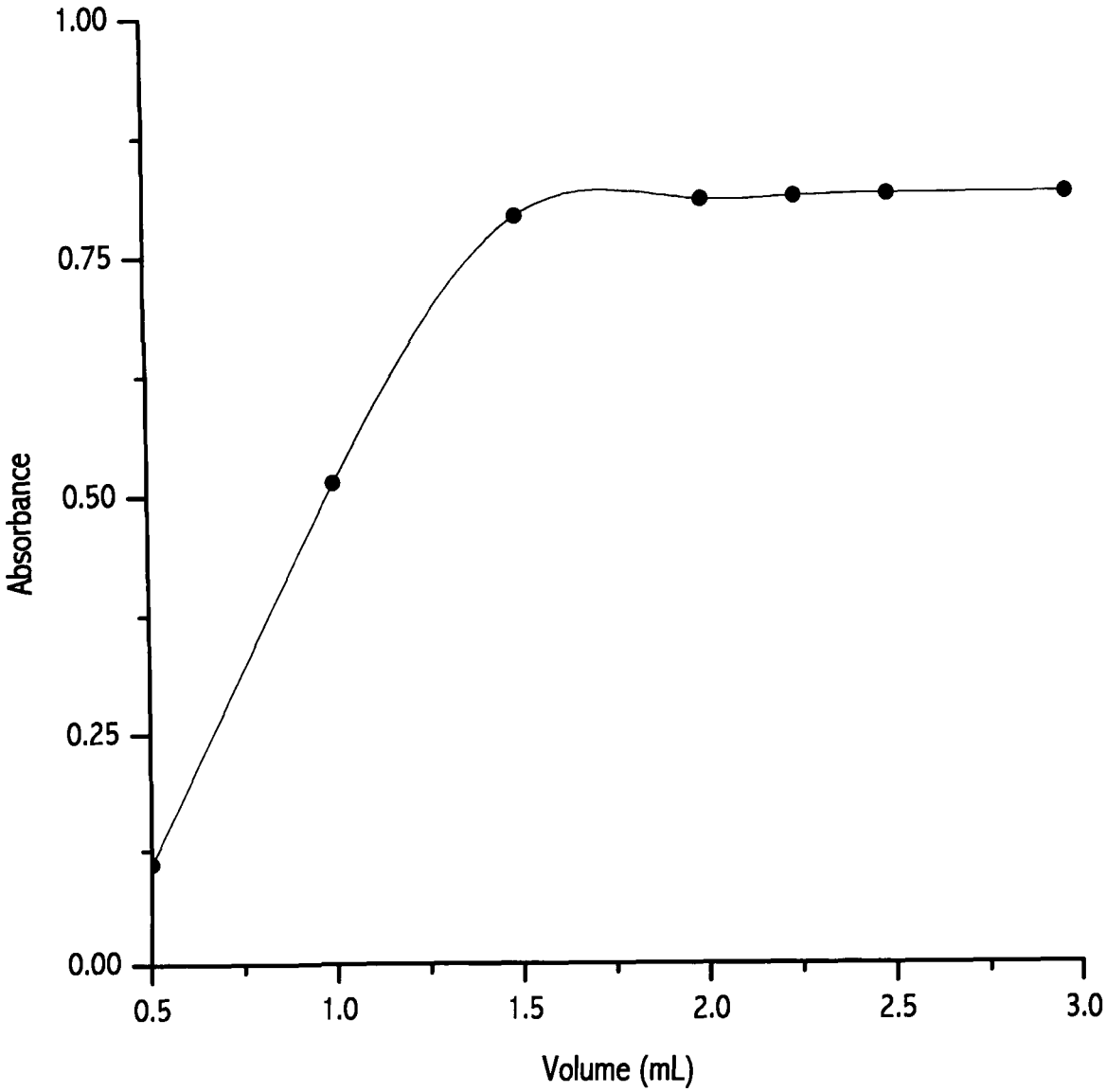


Fig. 3.3. Effect of volume of 1% chloramine–T on the oxidation of verapamil hydrochloride.

Effect of the concentration of hydrochloric acid

To study the effect of the concentration of hydrochloric acid, the reaction was carried out in a series of 10 mL volumetric flasks containing 200 μg mL^{-1} verapamil hydrochloride, 2.5 mL of 1% chloramine–T and varying volumes of 5 M HCl (2.0 – 6.5 mL). It is apparent from the Fig. 3.4 that the maximum absorbance was found with 5.8 mL of 5 M HCl, beyond which the absorbance became constant. Thus, 6 mL of 5 M HCl was used throughout the experiment.

Analytical data

Under the optimum experimental conditions, main merits of the procedure for the determination of verapamil hydrochloride have been established by least square treatment of the results. The absorbance responses at 425 nm were found to be linear in relation to the concentration of verapamil hydrochloride upto 340 μg mL^{-1} (Fig. 3.5) with a molar absorptivity of 2 x 10^3 L mol^{-1} cm^{-1}. Regression analysis of Beer's law plot was made to evaluate intercept, slope and correlation coefficient *(r)* and the values were found to be 0.29 x10^{-3}, 4.05 x10^{-3} and 0.9999, respectively which yielded the regression equation, $A = 0.29 \times 10^{-3} + 4.05 \times 10^{-3} C$ (where A is the absorbance and C is the concentration of verapamil hydrochloride in μg mL^{-1}). The detection limit for the proposed method was 0.97 μg mL^{-1} computed from the following equation [37].

$$\text{Detection limit} = \sqrt{S_0^2 \frac{n-2}{n-1}} \frac{t}{b}$$

where S_0^2 = variance, n = number of samples, t = Student's t–value at 95% confidence level and b = slope of the line of regression. The high value of correlation coefficient and small value of intercept on the ordinate, which was close to zero, validated the linearity of calibration curve

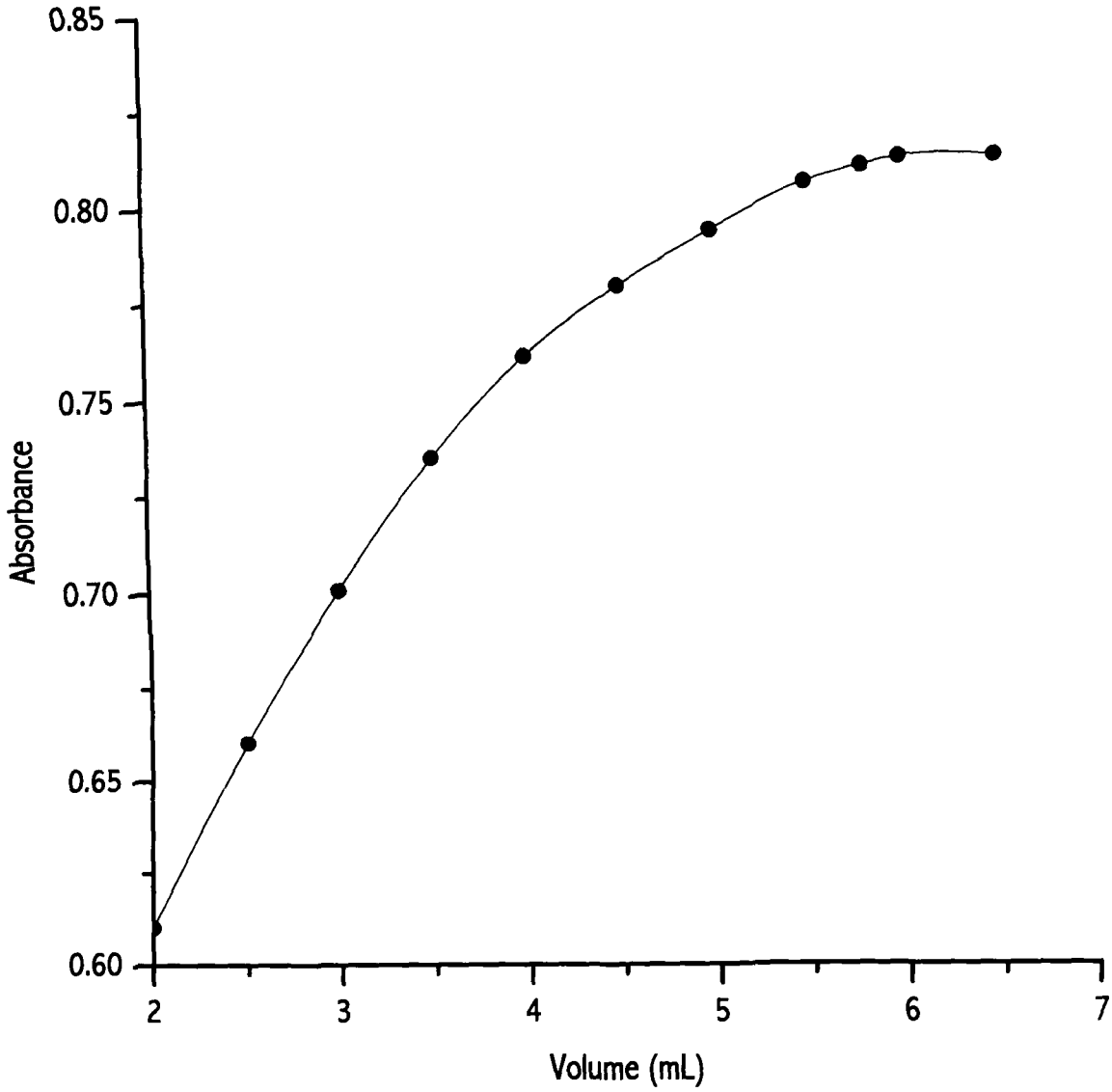


Fig. 3.4. Influence of the volume of 5 M hydrochloric acid on the oxidation of verapamil hydrochloride.

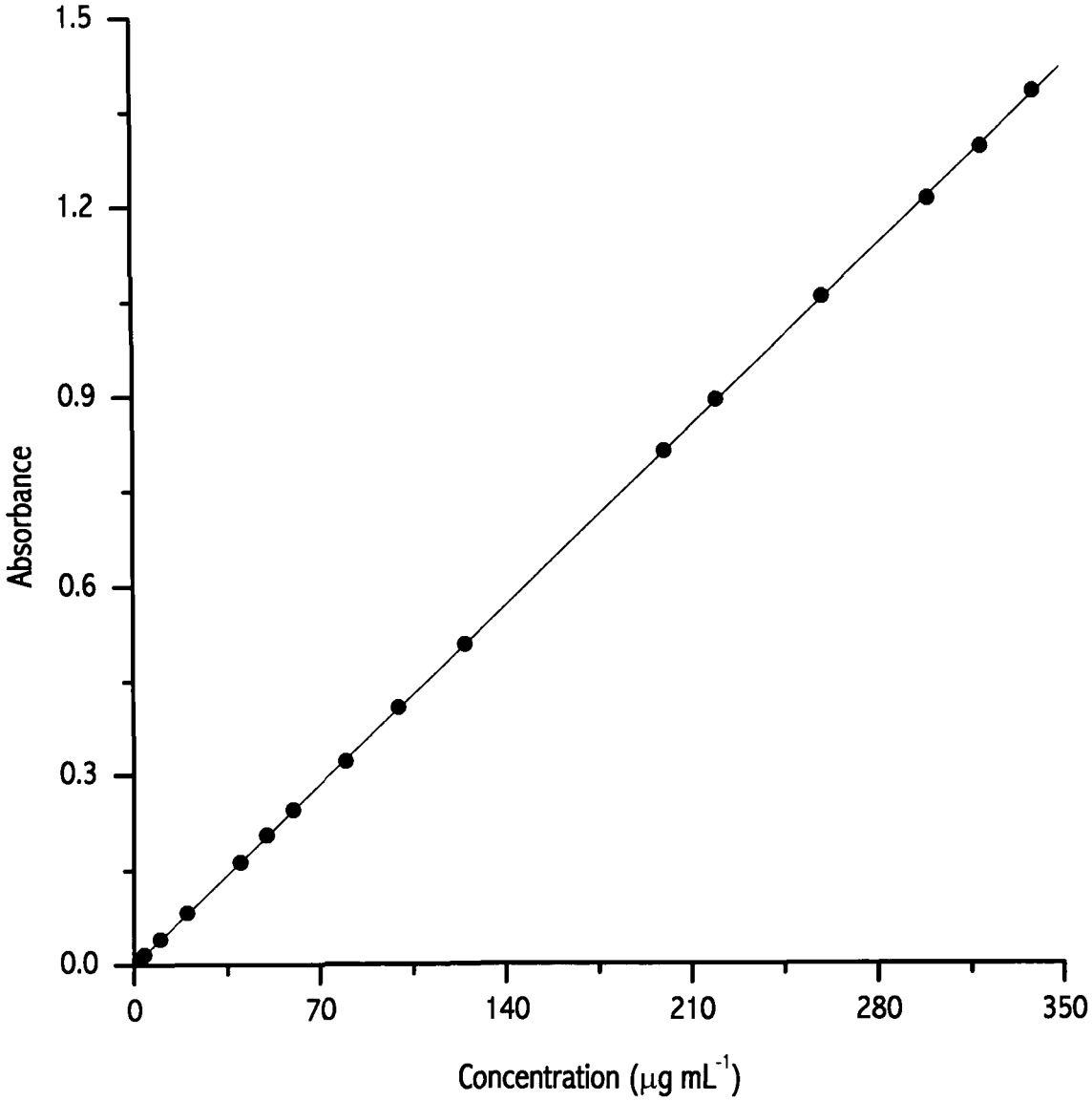


Fig. 3.5. Calibration curve for the determination of verapamil hydrochloride.

whereas detection limit and slope indicated the good sensitivity of the method. Also, the small degree of scattering of the experimental data points around the line of regression was confirmed by the small value of variance, i.e. 5.33 x10^{-6}.

There is also strong correlation between slope and intercept, which has been established by the 95% joint confidence region drawn for them [38]. It is evident from the Fig. 3.6 that the joint confidence region is bounded by an ellipse having the point of best fit as its centre. It can also be seen that the points with an intercept of zero fell well within the ellipse and thus, confirmed that there is no significant deviation from the zero.

Regression analysis of the calibration data also makes it possible to evaluate the error, S_c, in the determination of a given concentration of verapamil hydrochloride [39]. Fig. 3.7 shows the graph of S_c against the concentration of verapamil hydrochloride. The error is minimum when the actual absorbance become equal to the average absorbance value in the calibration graph which corresponds to 125 μg mL^{-1}. This statistical treatment may be used to establish the confidence limit at the selected level of confidence (Fig 3.8) for the determination of unknown concentration by using the equation [40].

$$C_l \pm \frac{t_p S_o}{b}\left[1 + \frac{1}{n} + \frac{(y - \bar{y})^2}{b^2(\Sigma C^2 - n\bar{C}^2)}\right]^{1/2} = C_l \pm \Delta C$$

In order to test the precision and accuracy of the proposed method ten successive determinations of 200 μg mL^{-1} of verapamil hydrochloride were carried out. The percent relative standard deviation (%RSD) and error (%Er) were found to be 0.24 and 0.22 respectively. The results, therefore, indicated that the method has satisfactory precision and accuracy. The commonly encountered execipients in the pharmaceutical dosage forms did not interfere.

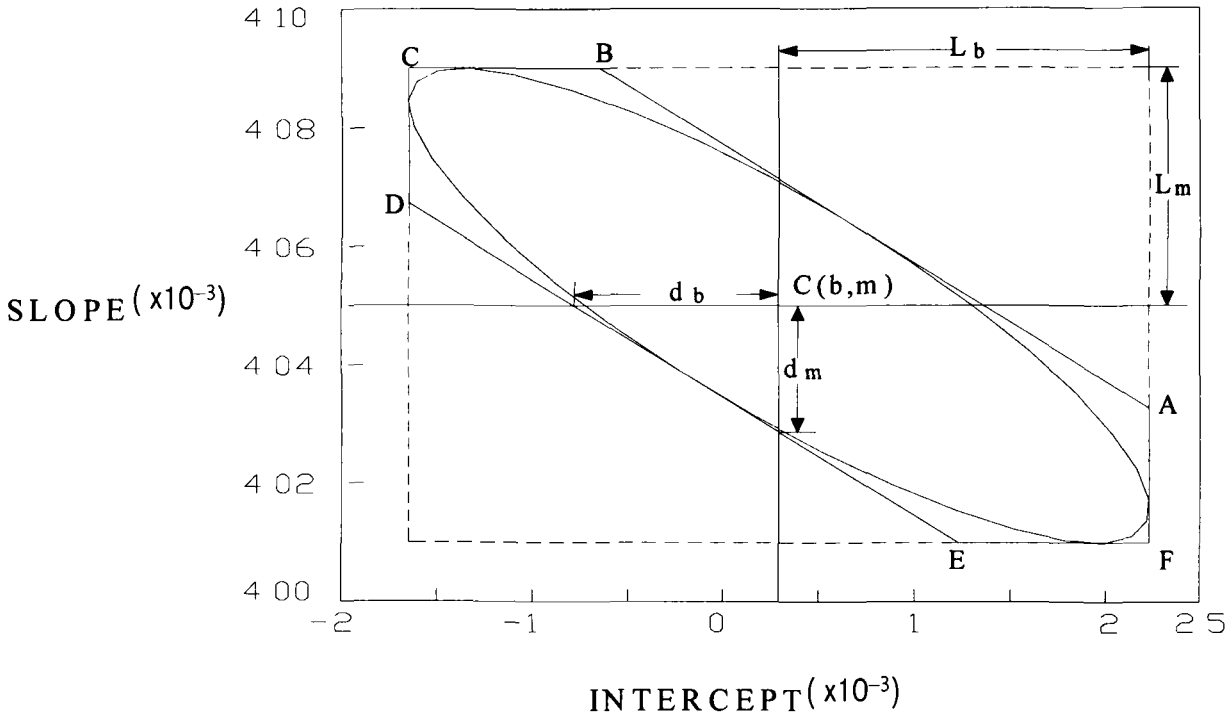


Fig. 3.6. Plot of joint confidence region (at P = 0.05) for the slope and intercept of the line of regression for the determination of verapamil hydrochloride.

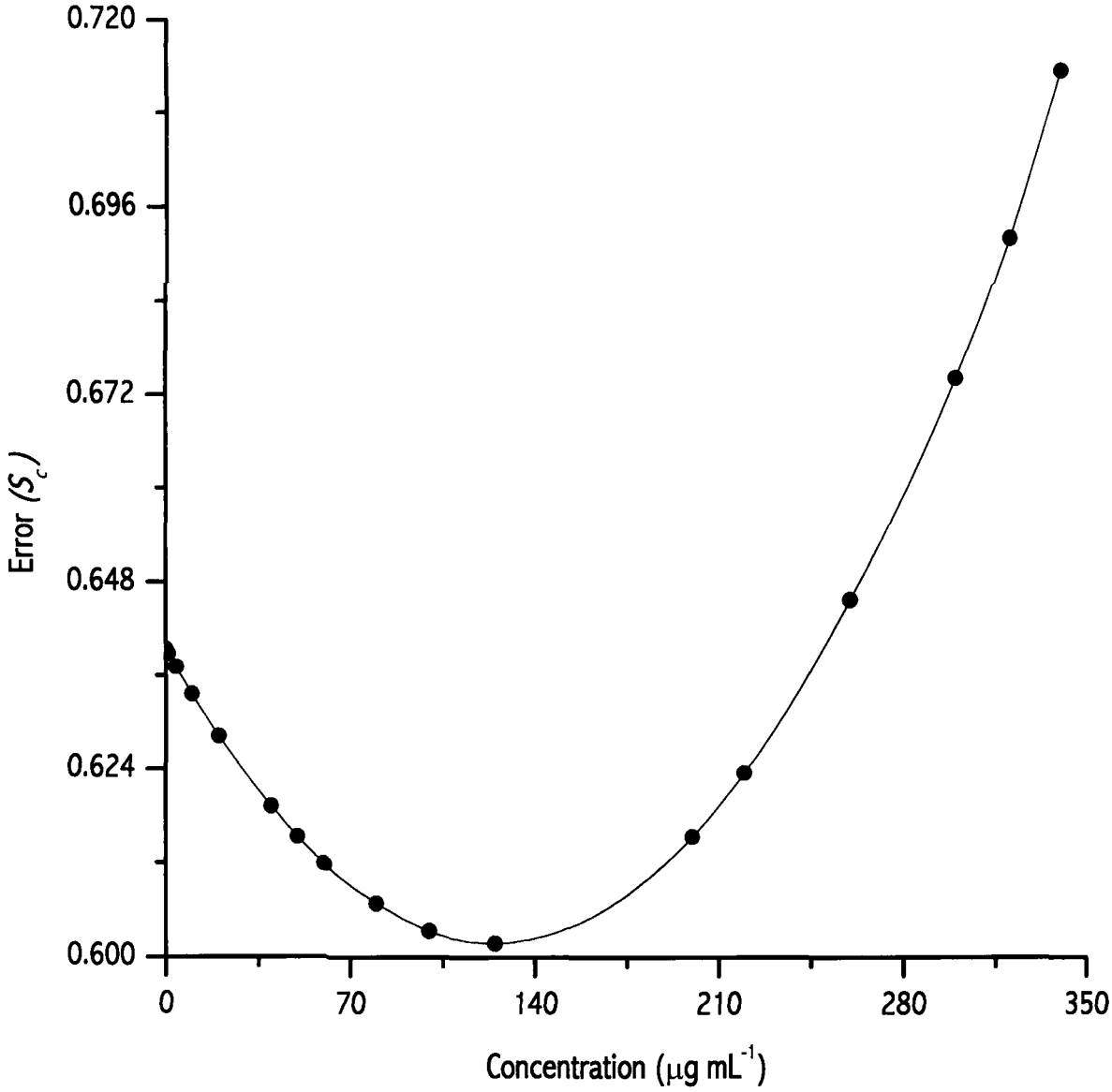


Fig. 3.7. Error *(Sc)* in the determination of the concentration of verapamil hydrochloride.

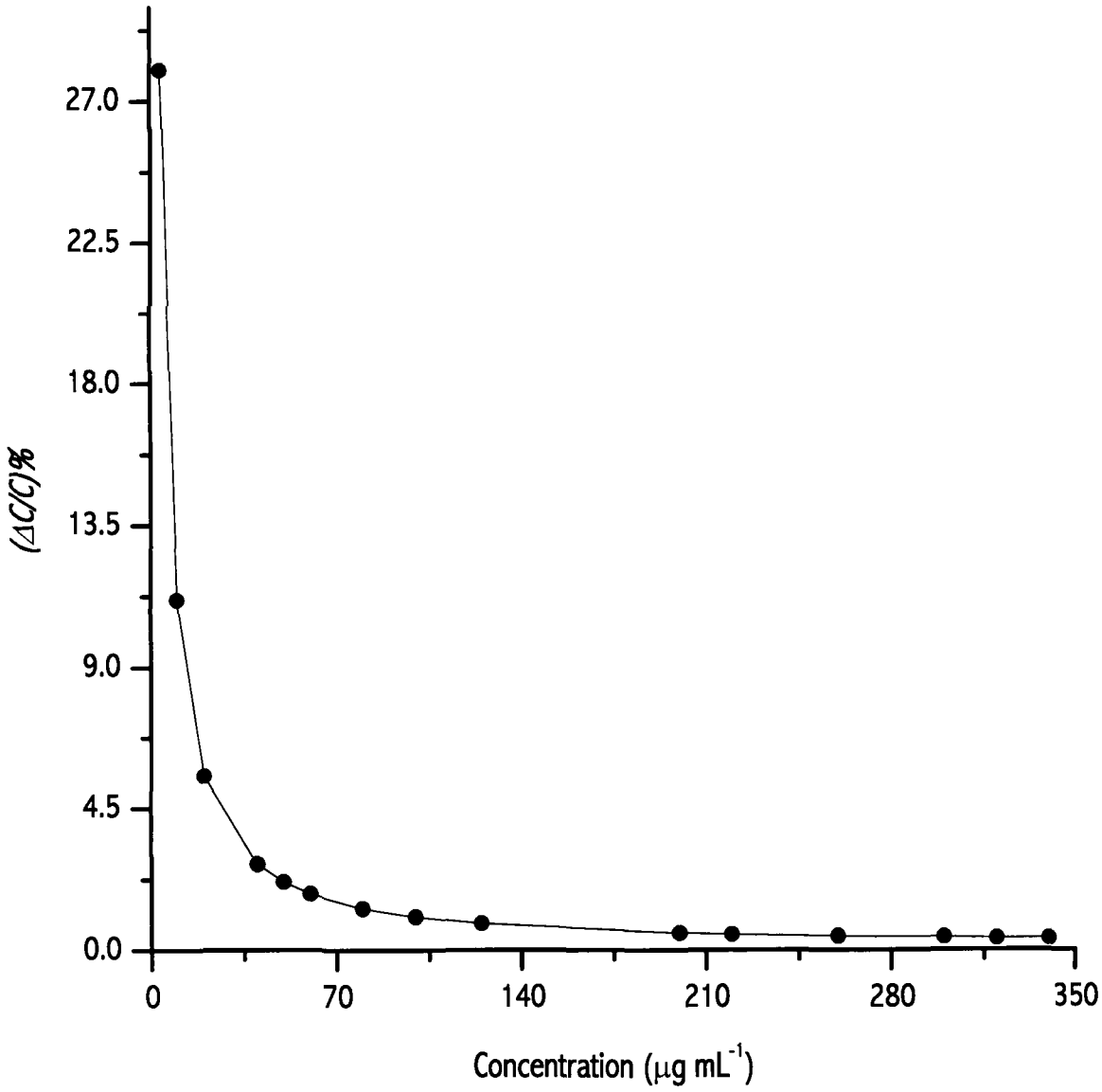


Fig. 3.8. Variation in the confidence limit at 95% confidence level for the determination of verapamil hydrochloride.

As an additional demonstration of accuracy, recovery experiments were carried out by adding a fixed amount of verapamil hydrochloride to a preanalysed tablet. The results are shown in Table 3.1. It is apparent from the table that results were reproducible with low percent relative standard deviations (0.3 – 0.82) and mean recoveries were in the range of 99.4 – 100.3%.

The method was successfully applied to the determination of verapamil hydrochloride in tablets available locally. Satisfactory results (Table 3.2) were obtained for the recovery of drug and were in good agreement with the label claimed. The results of the proposed method were statistically compared with those obtained by the reference method [22]. The calculated Student's *t*– and *F*–values were less than the theoretical ones at 95% confidence level. The statistical evaluation indicated that there was no significant difference between the methods compared.

The proposed method is advantageous when compared to other existing visible spectrophotometric methods in wider linear range of estimation with lower value of percent relative standard deviations (Table 3.3). This is a decisive advantage since commercial dosage forms contain higher amounts. The method is sensitive enough to permit the determination of as little as 0.97 $\mu g\ mL^{-1}$ of the drug. The proposed method, therefore, is simple, sensitive and reproducible and can be applied for the routine analysis of verapamil hydrochloride in the quality control laboratories.

Table 3.1. Determination of verapamil hydrochloride in dosage forms by standard addition method.

Pharmaceutical preparations	Amount taken ($\mu g\ mL^{-1}$)	Amount added ($\mu g\ mL^{-1}$)	Total amount found ($\mu g\ mL^{-1}$)[a]	Recovery (%)[a]	RSD (%)[a]
Calaptin-40	25	25	49.85	99.71	0.73
	150	150	300.20	100.10	0.30
Isoptin-40	25	25	50.15	100.30	0.82
	150	150	300.94	100.30	0.45
Vasopten-40	25	25	49.10	99.40	0.75
	150	150	300.2	100.10	0.67

[a] Five independent analyses.

Table 3.2. Analysis of pharmaceutical preparations by the proposed and reference methods.

Pharmaceutical Preparations	Labelled Amount (mg)	Proposed method		Reference method [22]		t_{calc}[b]	F_{calc}[b]
		Recovery (%)[a]	RSD (%)[a]	Recovery (%)[a]	RSD (%)[a]		
Calaptin	40	99.76	0.49	99.65	0.67	0.3154	1.9
Isoptin	40	100.12	0.41	99.88	0.49	0.8253	1.42
Vasopten	40	99.63	0.38	99.88	0.77	0.6720	4.16

[a] Average of five independent analyses.

[b] Theoretical *t*– and *F*–values at 95% confidence level are 1.86 and 6.39 respectively [41].

Table 3.3. Comparison of the proposed method with other spectrophotometric methods for the determination of verapamil hydrochloride in pharmaceutical formulations.

Reagent	λ_{max} (nm)	Beer's law limit ($\mu g\ mL^{-1}$)	RSD (%)	References
Erioglaucine[a]	627	1.3 – 5.3	1.45	[19]
Indigocaramine[a]	602	33 – 130	1.53	[19]
Bromocresol purple[a]	420	4 – 24	–	[17]
Chromotrope 2B	530	5 – 59	–	[23]
Chromotrope 2R	546	5 – 59	–	[23]
Solochrome dark blue[a]	528	10 – 38	1.53	[18]
Solochrome cyanine R[a]	445	8 – 30	1.24	[18]
Chloramine–T	425	0 – 340	0.3 – 0.82	This work

[a] Extractive method.

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Chapter - 4

Validated spectrophotometric methods for the determination of amlodipine besylate in drug formulations using 2,3-dichloro 5,6-dicyano 1,4-benzoquinone and ascorbic acid

Introduction

Amlodipine besylate is listed in *Martindale, The Extra Pharmacopoeia* and European Pharmacopoeia [1–2] which is chemically (4R,S)–3–ethyl 5–methyl 2–(2–amino–ethoxy–methyl)–4–(2–chlorophenyl)–1,4–dihydro–6–methylpyridine–3,5–dicarboxylate monobenzene sulphonate, is approved for the treatment of variant and stable angina and hypertension. It is relatively a new long acting calcium channel blocker with slow onset of vasodialatory action [3–4]. It may also be used for dilated cardiomyopathy and exhibits ameliorating effects on plasma and myocardial catecholamines with a significant reduction of calcium deposition [5–6]. In addition to calcium channel blocking ability, amlodipine also inhibits vascular smooth muscle cell growth through interactions with targets other than L–type calcium channels [7]. Amlodipine is more selective for arterial vascular smooth muscle than cardiac tissues. Due to these important pharmacological responses, development of sensitive and accurate methods for the determination of amlodipine besylate is desired.

Different methods for the quantification of amlodipine besylate have been reported which include high performance liquid chromatography [8–13], reversed phase high performance liquid chromatography [2,14–16], high performance thin layer chromatography [17–20], gas chromatography [21], gas chromatography–mass spectrometry [22], liquid chromatography with tandem mass spectrometry [23] and fluorimetry [24]. Though these methods are sensitive enough but are expensive and not easily manageable. On the other hand, spectrophotometry is still the technique of choice since it is sensitive, economical, rapid and more easily manageable for the third world countries.

Few spectrophotometric methods have been reported for the assay of amlodipine besylate based on extractable ion-pair complexes [25–29], oxidative coupling with 3–methyl 2–benzothiazolinone hydrazone hydrochloride [30], with sodium hydroxide [31], derivative spectroscopy [32–33], simultaneous multicomponent mode of analysis [34] and charge transfer complexation with p–chloranilic acid [35] and chloranil [36]. It has also been determined based on the reaction of $-NH_2$ group with ninhydrin in drug formulations [37].

A literature survey of charge transfer complexation reactions of polyhalo/ polycyanoquinones with basic nitrogenous centres reveals that 2,3–dichloro 5,6–dicyano 1,4–benzoquionone (DDQ) is one of the sensitive reagents among them which acts as an electron acceptor and yields more sensitive results in comparison to other polyhaloquinones [38–41].

In 1964, Jaroslav Bartos introduced ascorbic acid as a sensitive and economical reagent for the detection and determination of primary amino group in N, N–dimethyformamide (DMF) medium [42]. Since then the mechanism of this reaction has not been exactly elucidated yet, so no much attention have been paid to the use of ascorbic acid as an economical spectrophotometric reagent for the determination of amino group specially in pharmaceutical analysis.

This chapter describes two sensitive, fast, simple and economical methods for the determination of amlodipine besylate in pure and dosage forms. The first method is based on the charge transfer complexation reaction of amlodipine with DDQ. The second procedure utilises the reaction of primary $-NH_2$ group of the drug with ascorbic acid in DMF medium. The proposed methods are validated statistically.

Experimental

Reagents and materials

All chemicals used were of AR–grade. Water was doubly distilled. A 0.1% solution of amlodipine besylate (Wockhardt Ltd., India) was prepared in chloroform. 0.1% solution of amlodipine besylate was also prepared in N, N–dimethylformamide (S.D. Fine Chem. Ltd., India). As reagents 0.05% DDQ (Fluka, Switzerland) solution in acetonitrile and 0.5 M aqueous sodium carbonate (E. Merck, India) solution were prepared for DDQ method. For the second method, 0.2% ascorbic acid was prepared by dissolving 100 mg of ascorbic acid (S.D. Fine) in 0.5 mL of water in a 50 mL standard flask and diluting to the volume with DMF.

Recommended Procedures

DDQ Method

Preparation of amlodipine base solution

In a 150 mL separatory funnel, 50 mL of 0.1% amlodipine besylate solution in chloroform was transferred followed by 75 mL of 0.5 M aqueous sodium carbonate solution. The content was mixed well and shaken for few minutes. The organic layer was separated and dried over anhydrous sodium sulphate. A 25 mL portion of organic layer containing amlodipine base was evaporated to dryness and the residue was taken up with acetonitrile and transferred to 50 mL standard volumetric flask, diluting to volume. This corresponds to 0.05% amlodipine base solution.

Procedure for the determination

Aliquots of 0.05% amlodipine base corresponding to 1 – 125 μg mL^{-1} were transferred into a series of 5 mL volumetric flasks. 1.0 mL of 0.05% DDQ solution was added

in each flask and diluted to volume with acetonitrile. The coloured product formed immediately and remained stable from 3 – 25 minutes. Therefore, the absorbances were measured within the stability period at 580 nm against the reagent blank prepared simultaneously.

Ascorbic Acid Method

Into a series of boiling tubes, aliquots of 0.1% amlodipine besylate solution in DMF (10 – 140 μg mL^{-1}) were pipetted. To each tube, 2.5 mL of 0.2% ascorbic acid solution was added. The total volume in each tube was maintained to 5 mL by adding DMF. The contents were mixed well and placed on a water bath maintained at 100±1 °C for 25 minutes. The solutions were cooled to room temperature. The reaction mixture and their corresponding washings were transferred and collected into a series of 10 mL volumetric flasks. They were diluted to volume with DMF. The absorbances were measured within the stability period of 4 hours at 530 nm against the reagent blank prepared simultaneously omitting the drug.

Analysis of Pharmaceutical Formulations

Ten tablets (claiming for 10 mg of amlodipine besylate per tablet) were finely powdered and extracted into sufficient volume of chloroform with shaking. The residue was filtered on whatmann filter paper no. 42 and the filtrate was diluted to 100 mL with chloroform. The solution of amlodipine base was prepared as discussed above and the drug was analysed following the recommended procedure using DDQ solution as the reagent.

An accurately weighed portion of powdered tablets equivalent to 100 mg of amlodipine besylate was stirred with sufficient volume of DMF and left standing for 10 minutes. The residue was filtered on whatmann filter paper no. 42 and washed well with DMF. The filtrate and

washings were diluted to volume into a 100 mL volumetric flask. The assay was completed following the recommended procedure using ascorbic acid solution as the reagent.

Results and discussion

Reaction mechanism and IR studies

The molecular interactions between electron donors and acceptors are generally associated with the formation of intensely coloured charge transfer complexes or radical anions depending on the polarity of the solvent used. DDQ is a π-acceptor which readily forms charge transfer complexes with basic nitrogenous compounds as n-donors [38–41]. Some salts of amines do not react with σ- or π- acceptors because of non-availability of lone pair of electrons. In a similar manner, amlodipine besylate does not react with DDQ. In order to determine amlodipine besylate, the drug was dissolved in chloroform and shaken with a 0.5M aqueous sodium carbonate solution. This treatment yielded amlodipine base in chloroform layer and evaporated to dryness. The residue was taken up in acetonitrile, a more polar solvent. The amlodipine base acts as an n-donor to form reddish violet coloured charge transfer complex with DDQ showing absorption maxima at 435, 550 and 580 nm (Fig. 4.1). These bands may be attributed to the formation of DDQ radical anions, which probably resulted through the dissociation of the donor–acceptor complex in a highly polar solvent like acetonitrile. In order to avoid the maximum interference from the reagent blank, the absorption band at λ_{max} 580 nm was chosen for the analytical studies. The Job's plot (Fig. 4.2) suggested a donor to acceptor ratio of 1 : 2, confirming the presence of two n-donating centres in the amlodipine `base molecule [47]. On the basis of the literature background and our experimental observations, a reaction mechanism is proposed and given in Scheme 4.1.

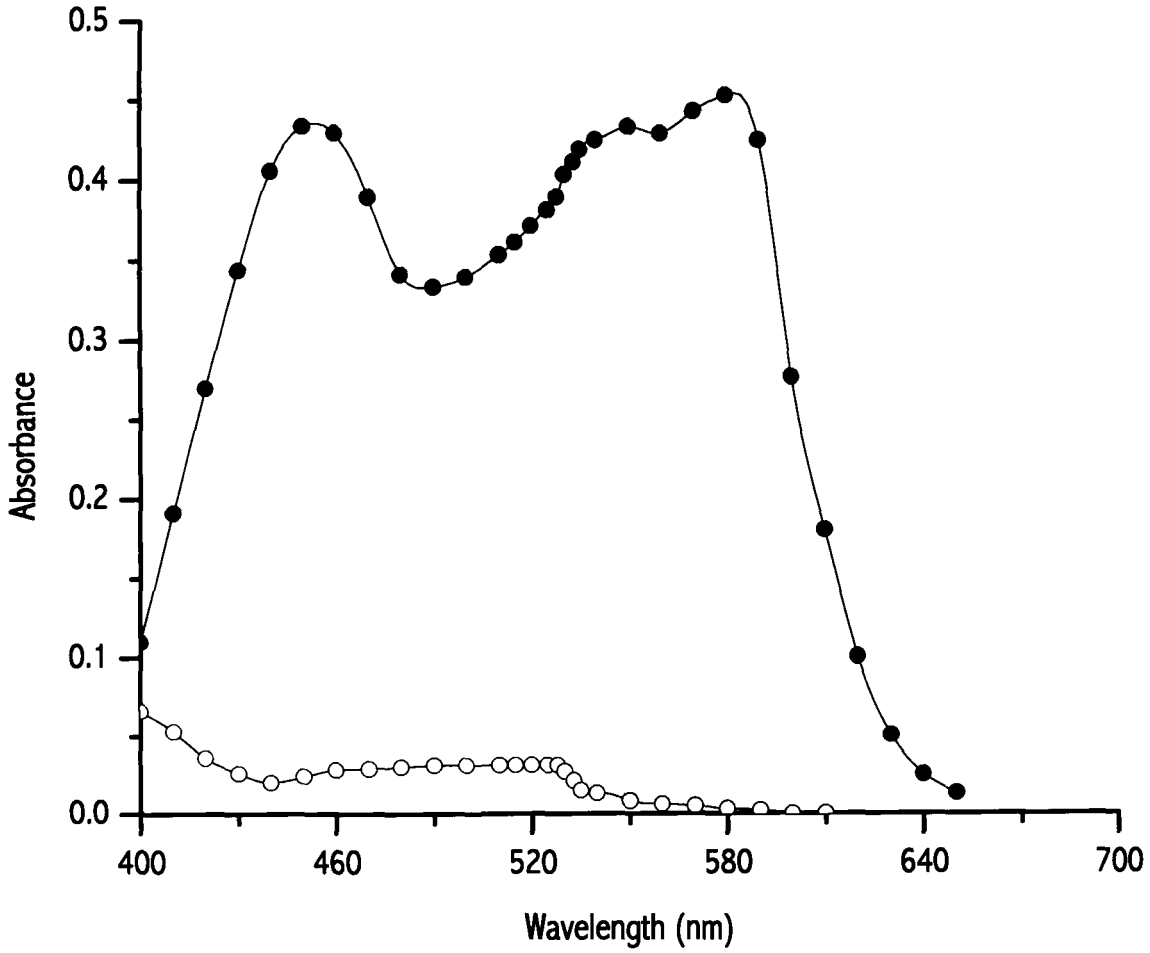


Fig 4.1. Absorption spectra of the reaction product of amlodipine with DDQ (•) and its reagent blank (o).

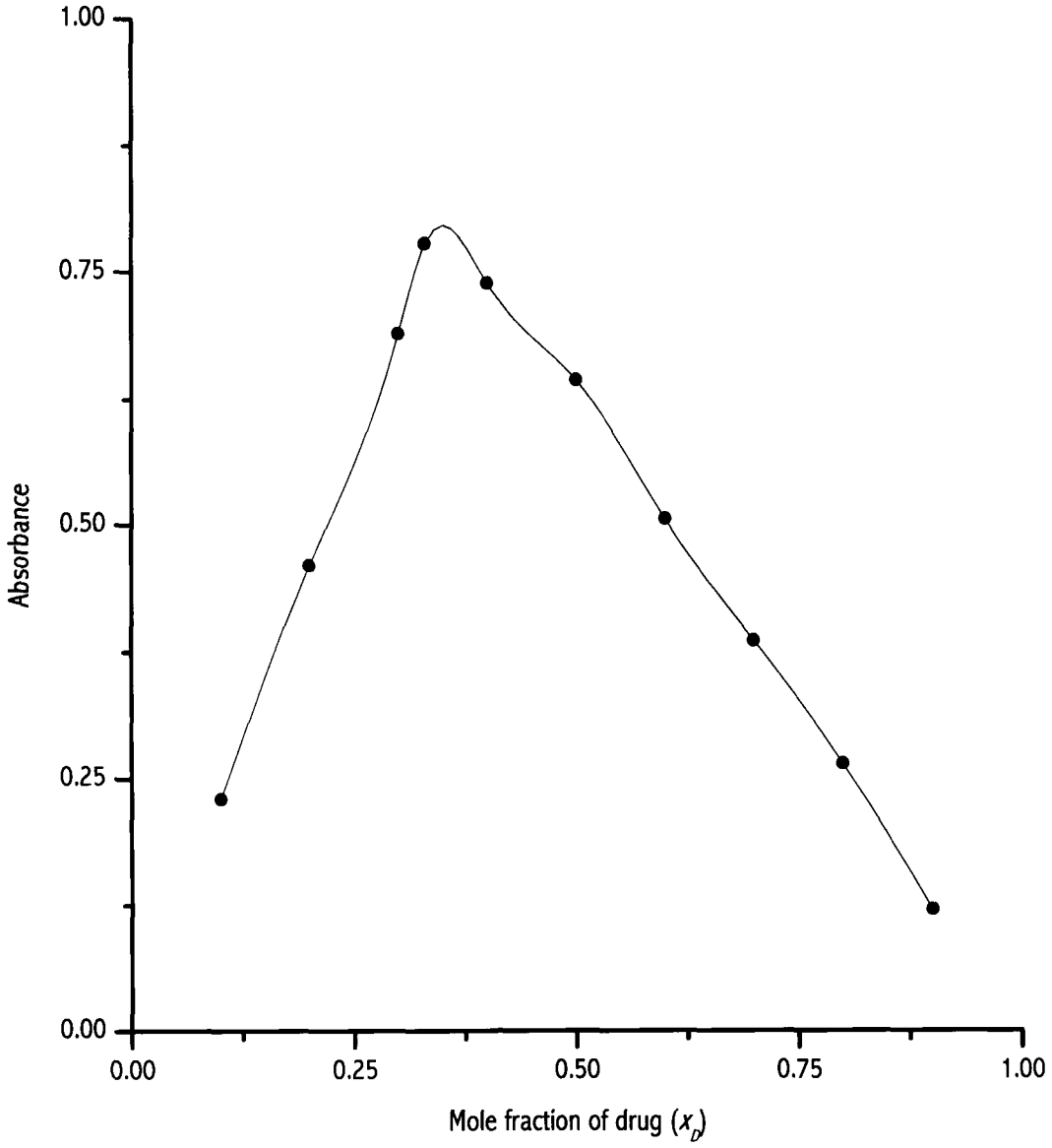
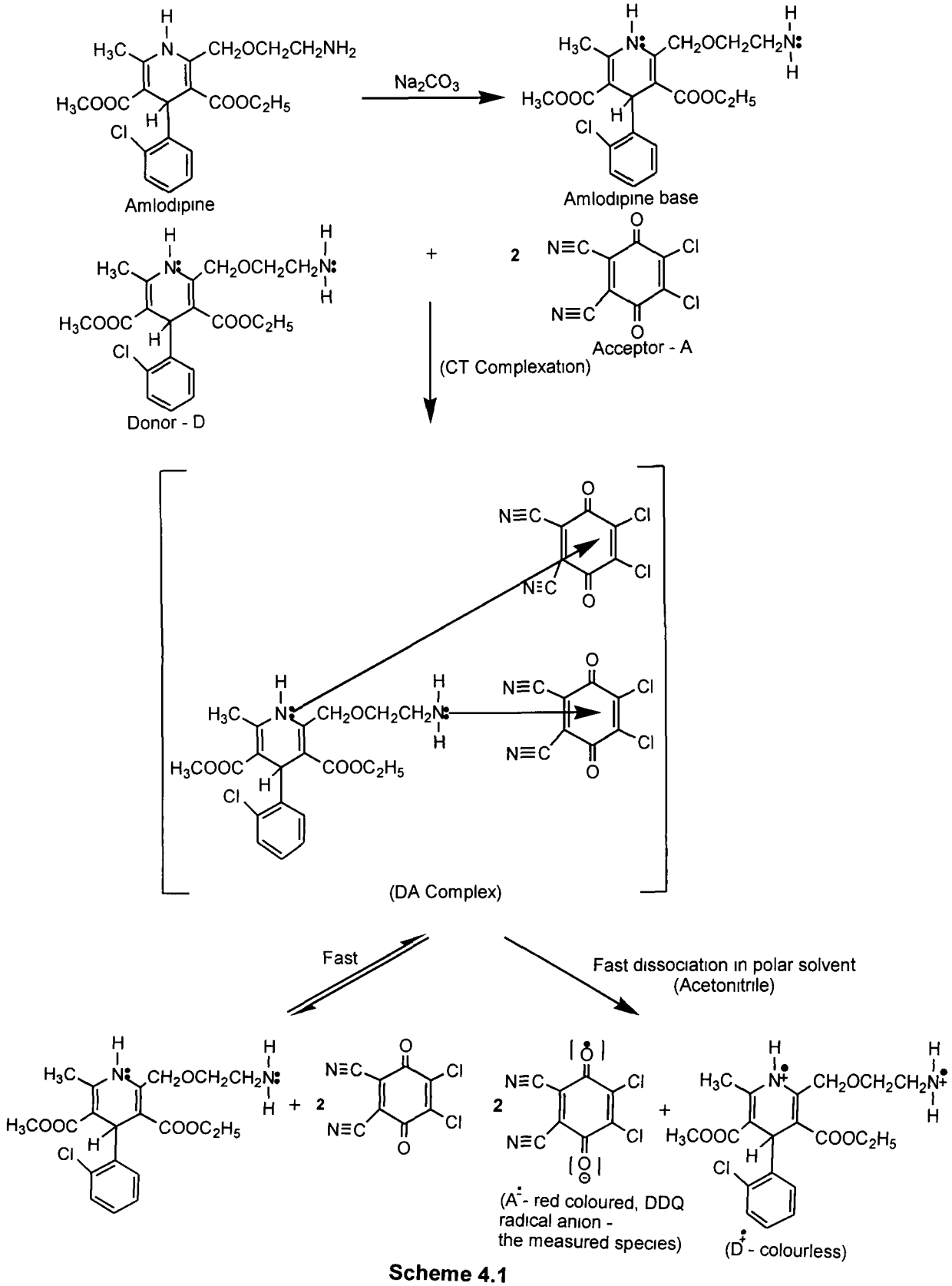


Fig. 4.2. Job's plot for amlodipine–DDQ complex.


Amlodipine
Na2CO3
Amlodipine base
Donor - D
2
Acceptor - A
(CT Complexation)
(DA Complex)
Fast
Fast dissociation in polar solvent
(Acetonitrile)
(A•- red coloured, DDQ
radical anion -
the measured species)
(D• - colourless)

Scheme 4.1

A purple red coloured product is obtained on heating amlodipine besylate with ascorbic acid in DMF, which absorbed maximally at 530 nm (Fig. 4.3). On heating in water-bath, the oxidation of ascorbic acid occurs mainly due to the formation of dehydroascorbic acid [48]. The carbonyl group further reacts with $-NH_2$ group of amlodipine to form a purple red coloured imine. The IR spectrum of amlodipine besylate (Fig. 4.4) displayed a band in the region 3120 – 2950 cm^{-1} attributed to N^+–H stretching mode. A sharp band at 1697 cm^{-1} may be due to C=O stretching vibration. The aromatic nature of the drug is characterized by the bands appearing in the region 1610 – 1450 cm^{-1}.The band at 1303 cm^{-1} and other bands in the region 1200 – 1000 cm^{-1} may be assigned to C–N (ring) and C–N (aliphatic amine) stretching vibrations [49]. The IR spectrum of the reaction product (Fig. 4.5) exhibits a broad band in the region 3600 – 3300 cm^{-1} which may be attributed to –OH and –CH stretching vibrations wherease N^+–H stretching mode disappeared. Another sharp and strong band at 1670 cm^{-1} suggested the formation of C=N group. The C=O stretching vibration is also shifted to a lower value and may appear in the C=N group region. The IR spectrum of the product has also indicated the OH deformation vibrations and the presence of C–O–C stretching appearing at 1256 and 1102 cm^{-1}, respectively. Thus the comparative study of the IR spectra of amlodipine besylate and the reaction product suggested an imine formation. The reaction mechanism is proposed in Scheme 4.2.

Optimization of Variables

In DDQ method, the only effective variable is the concentration of DDQ since the reaction gets stabilised within 3 minutes and remains unaffected for further 20 minutes. To study the effect of concentration of DDQ, varying volumes of 0.05% reagent were mixed with

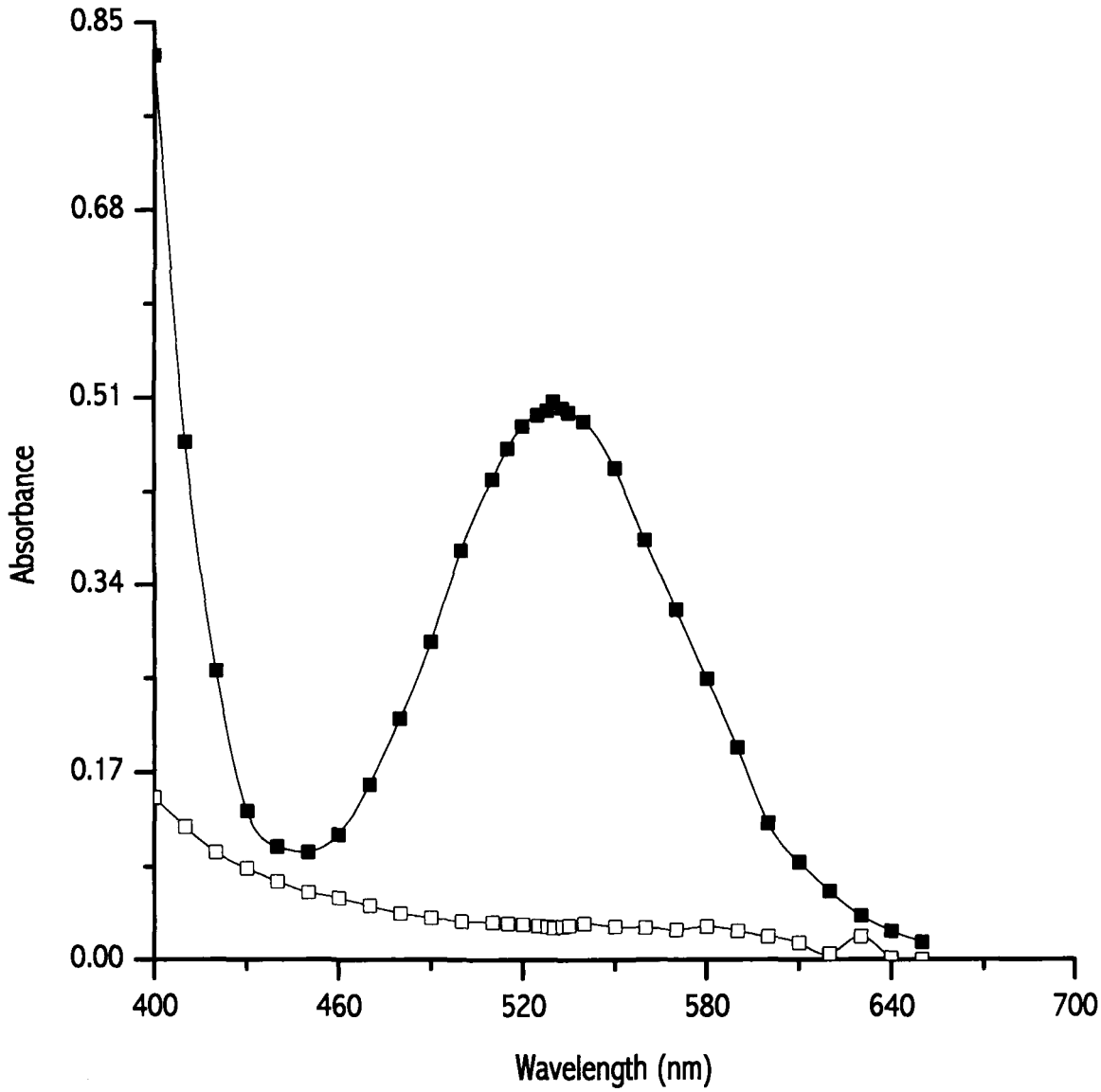


Fig. 4.3. Absorption spectra of the reaction product of amlodipine besylate with ascorbic acid in DMF (■) and its reagent blank (□).

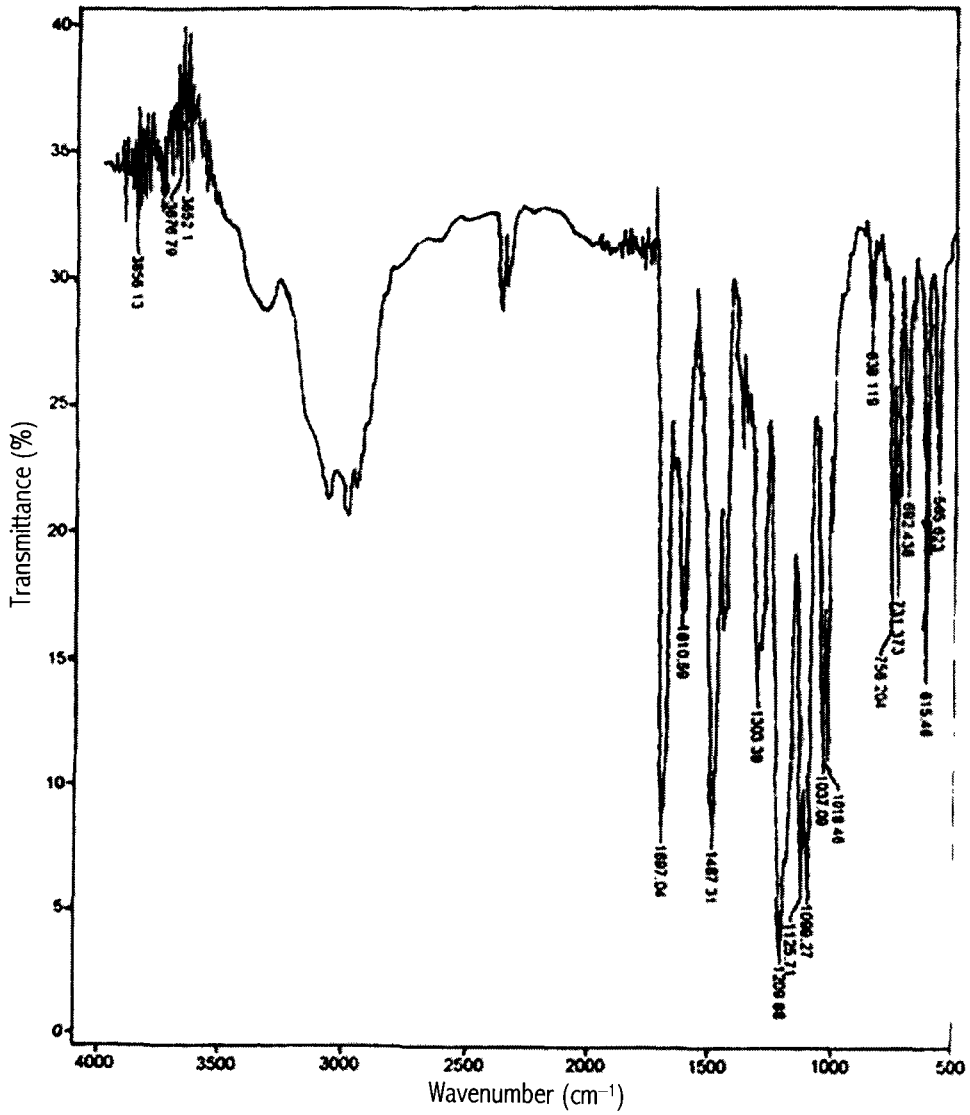


Fig. 4.4. FTIR–spectrum of amlodipine besylate.

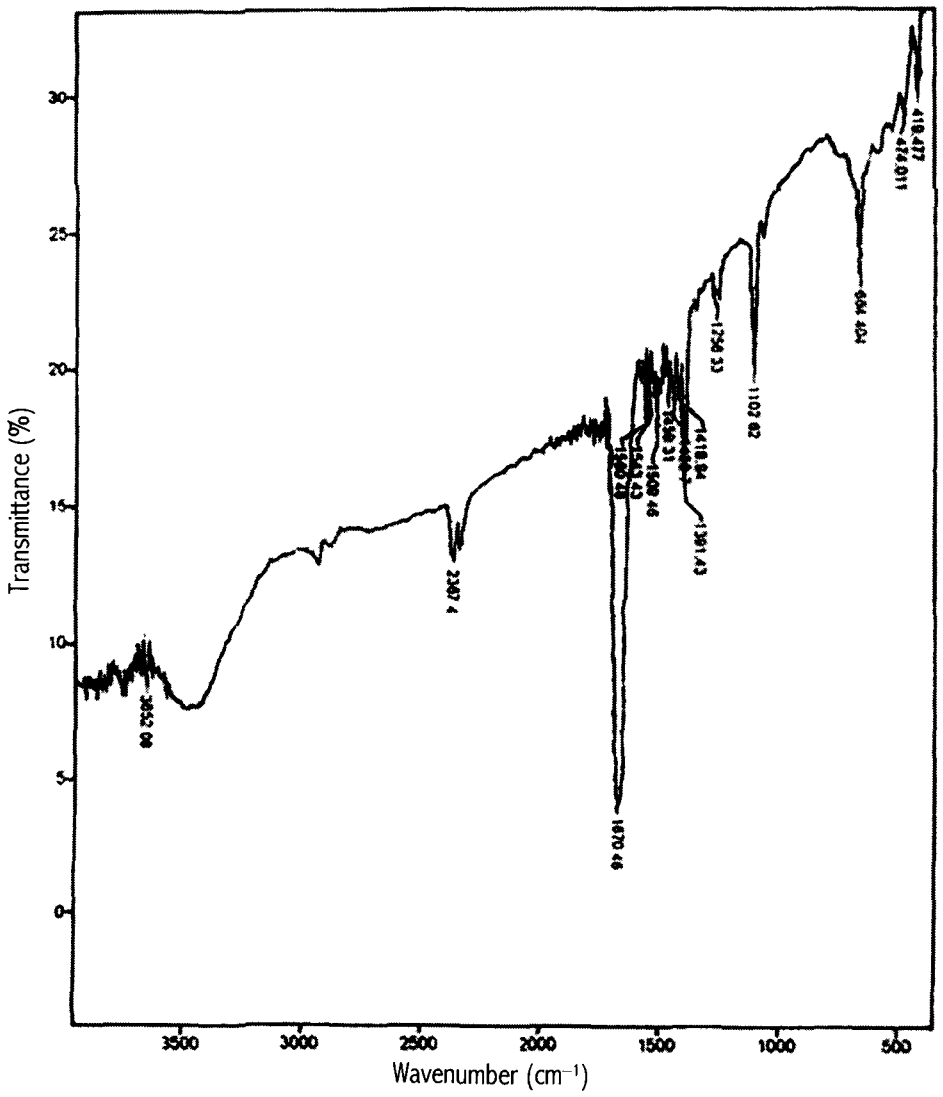
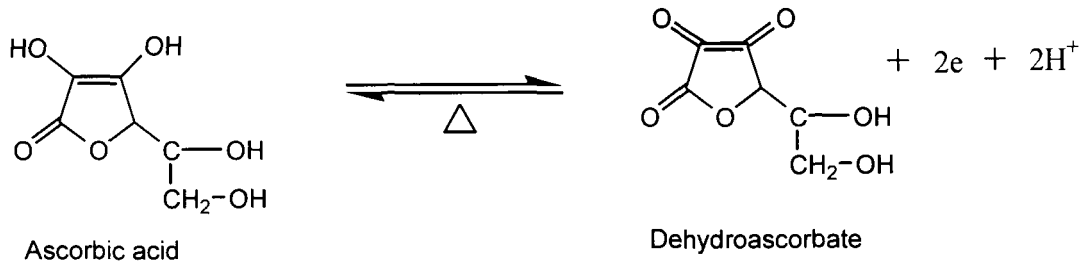
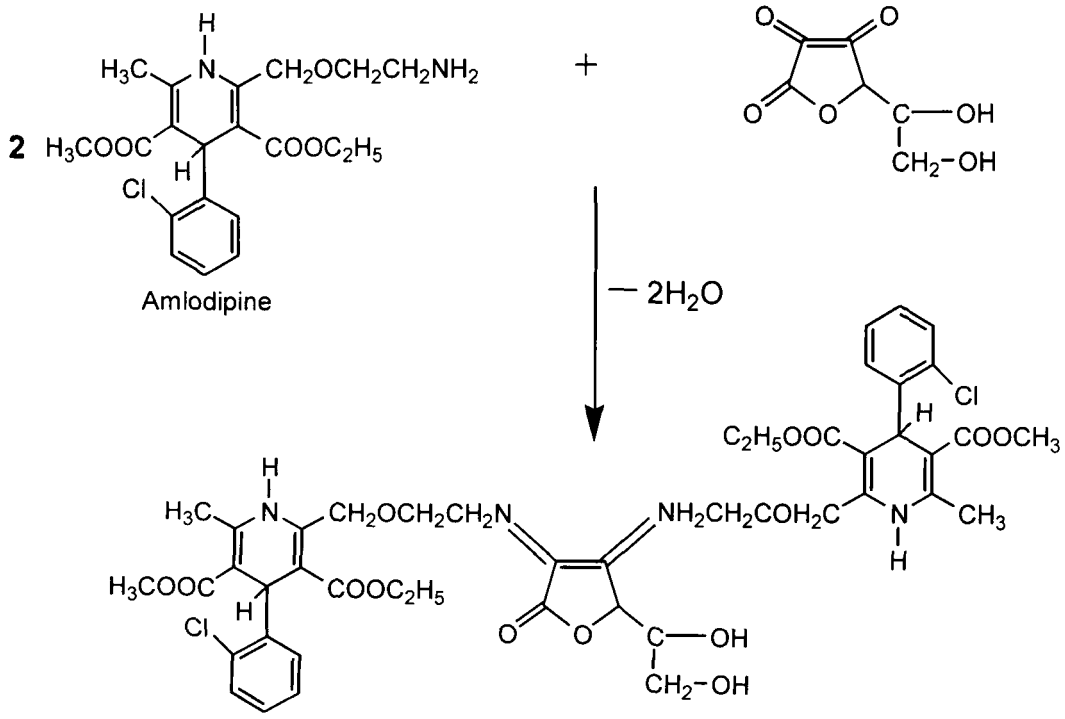


Fig. 4.5. FTIR–spectrum of the reaction product of amlodipine besylate–ascorbic acid.

I. Formation of dehydroascorbate from ascorbic acid.



II. Imine formation by the coupling of amlodipine with dehydroascorbate.



Scheme 4.2

0.5 mL of drug in a 5 mL standard flask and diluted to volume with acetonitrile. The absorbances were measured after 3 minutes of mixing at 580 nm against the corresponding reagent blanks. It was found that 0.5 mL of the reagent gave the highest absorbance (Fig. 4.6); above this volume the absorbance remains constant. A volume of 1.0 mL was, therefore, used in all further measurements.

To optimize the heating time for ascorbic acid method, 1.0 mL of 0.1% amlodipine besylate was mixed with 2.5 mL of 0.2% ascorbic acid and heated at 100±1^{0}C. The absorbance was measured at 530 nm against the reagent blank as a function of heating time. The results (Fig. 4.7) show that the absorbance remains constant between 22 and 32 minutes of heating. There is an abrupt change in the absorbance above 32 minutes of heating and therefore, 25 minutes of heating time was used throughout the experiment.

In order to study the effect of volume of reagent on the absorbance, varying volume of 0.2% ascorbic acid was mixed with 1.0 mL of 0.1% amlodipine besylate in different boiling tubes and the contents were heated on the water bath at 100±1 °C for 25 minutes. The highest absorbance was obtained with 1.75 mL of the reagent (Fig. 4.8); above which the absorbance remains unaffected. A 2.5 mL of the reagent was taken as the optimum value.

Analytical Data

Under the optimum experimental conditions, linear calibration graphs were obtained over the concentration ranges 1 – 125 and 10 – 140 μg mL^{-1} (Fig. 4.9 and 4.10) of amlodipine besylate with molar absorptivities of 0.60 x10^4 and 0.32 x10^4 L mol^{-1} cm^{-1} using DDQ and ascorbic acid, respectively. The calibration data were fitted by least square treatment and the regression equations obtained for DDQ and ascorbic acid methods were $A = 3.54 \times 10^{-3} + 8.88 \times 10^{-3} C$

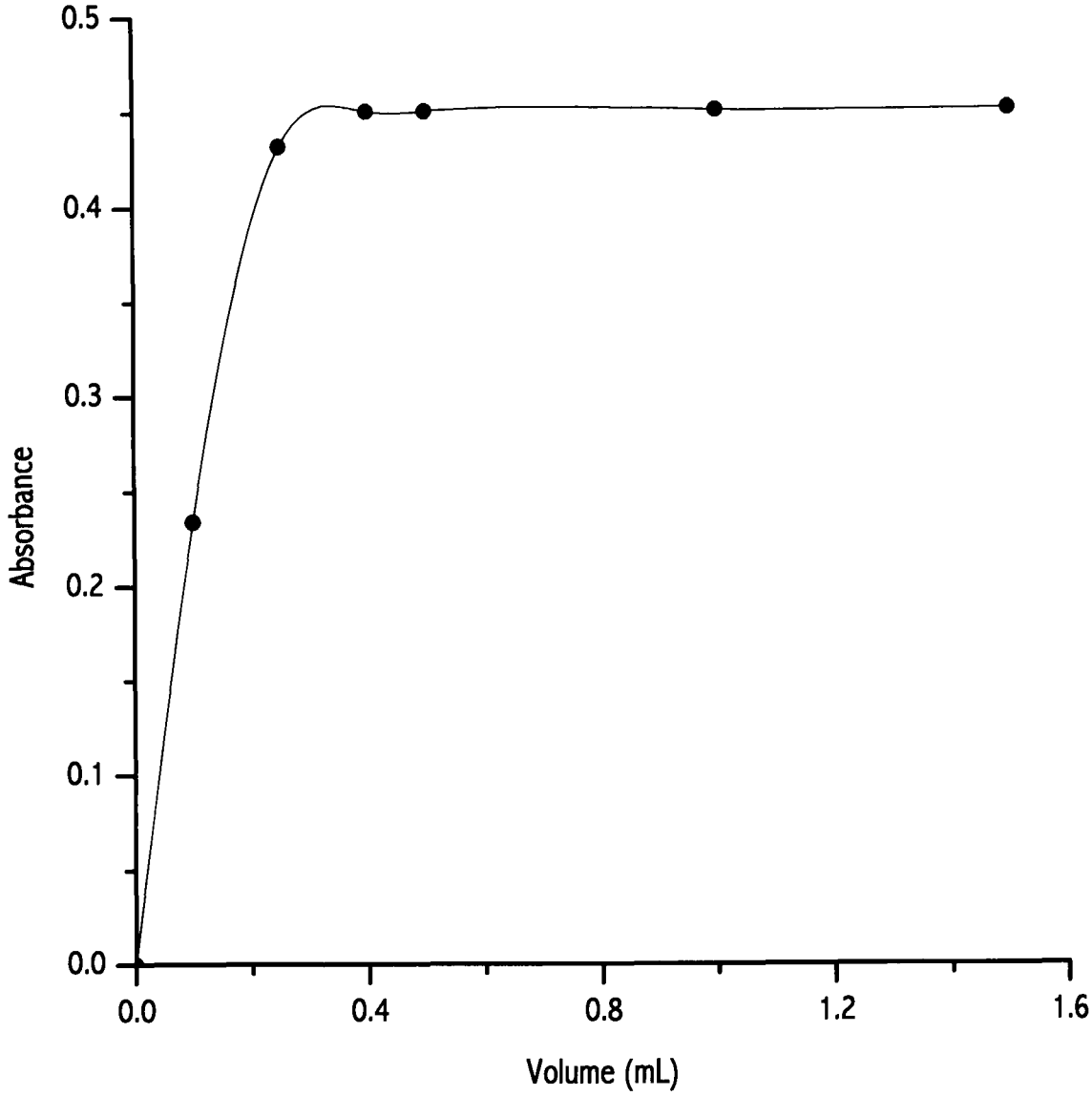


Fig. 4.6. Effect of the volume of 0.05% 2,3–dichloro 5,6–dicyano 1,4–benzoquinone solution.

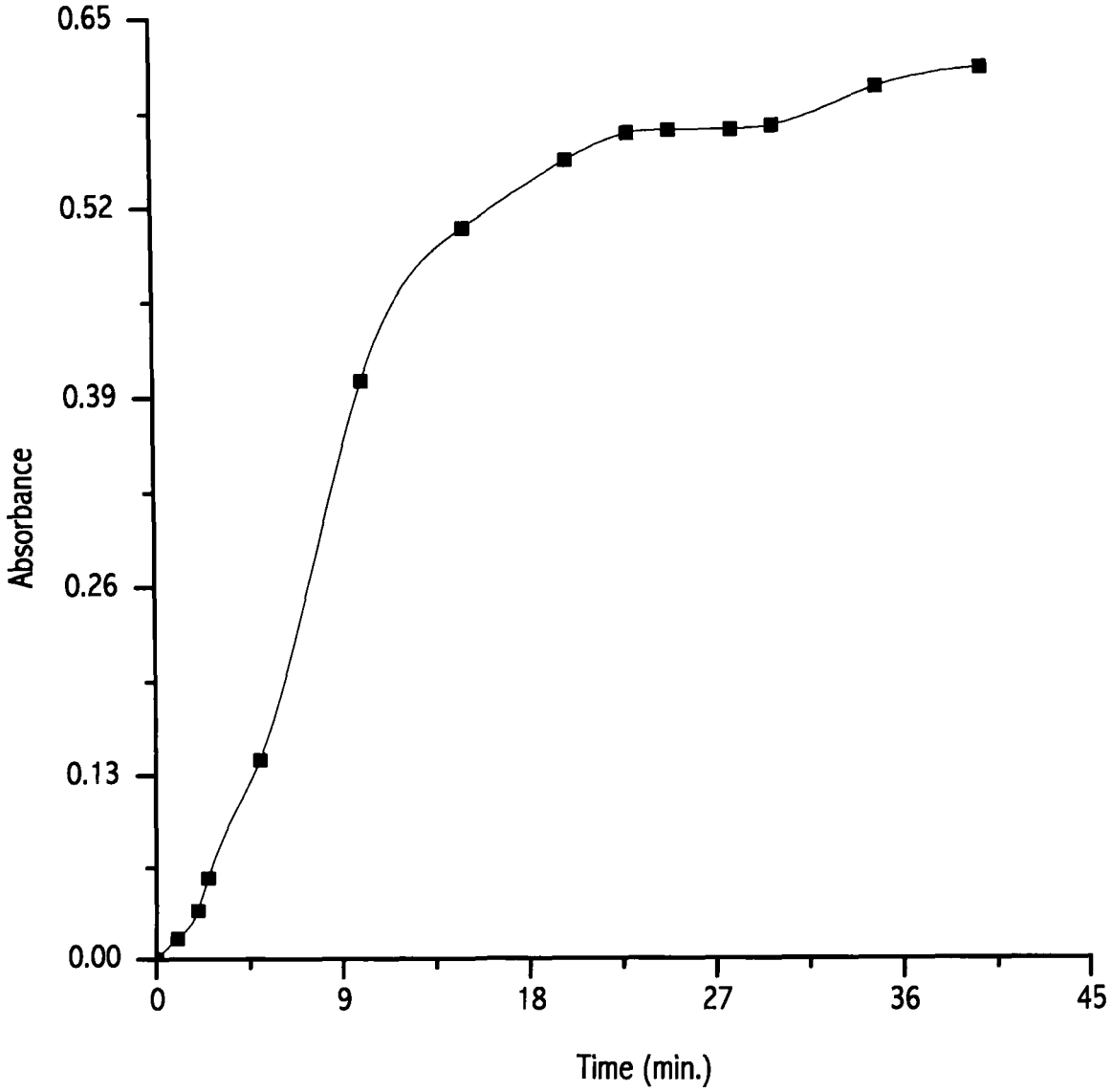


Fig. 4.7. Effect of the heating time on the reaction of amlodipine with ascorbic acid in DMF.

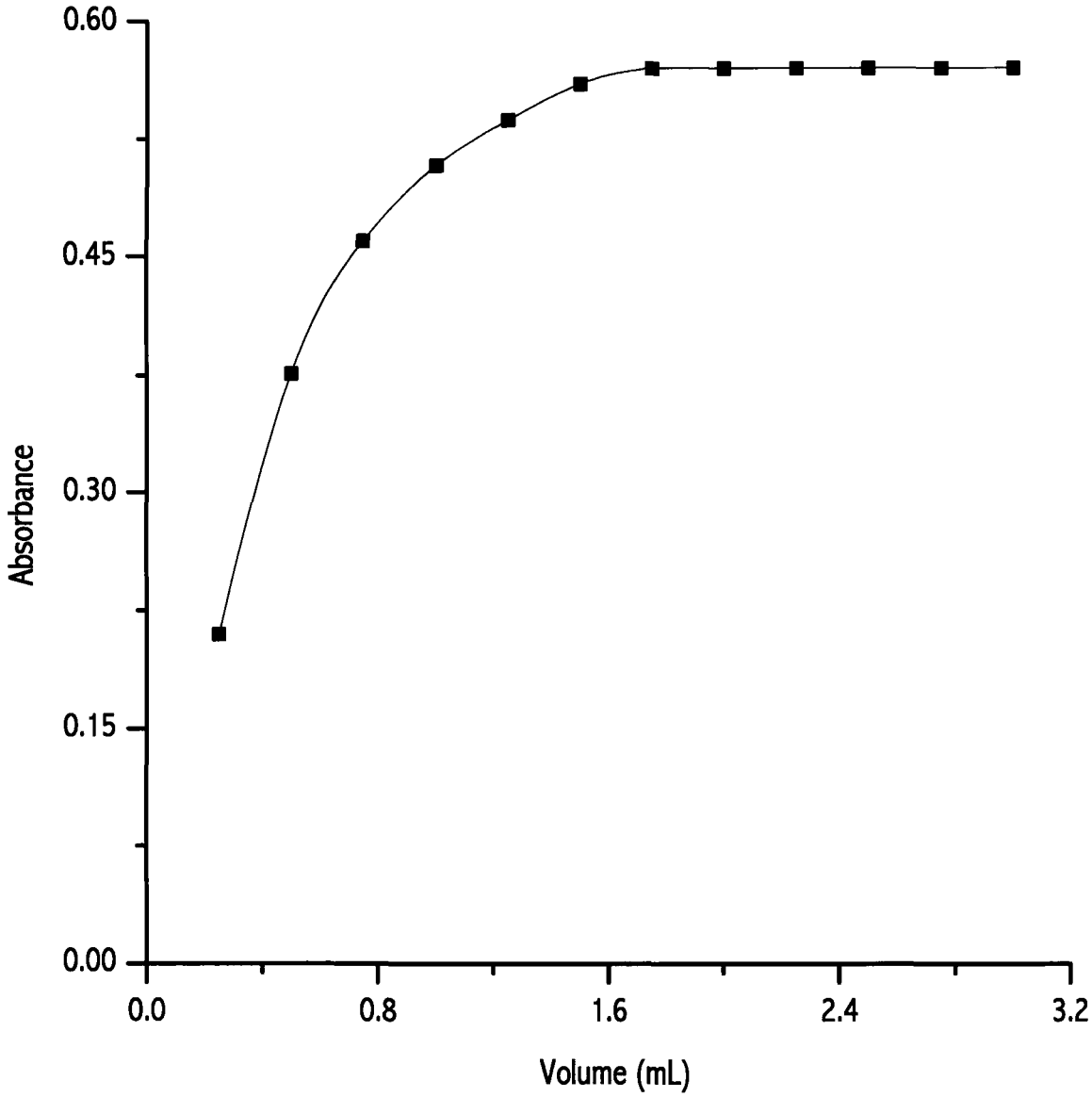


Fig. 4.8. Effect of the volume of 0.2% ascorbic acid solution.

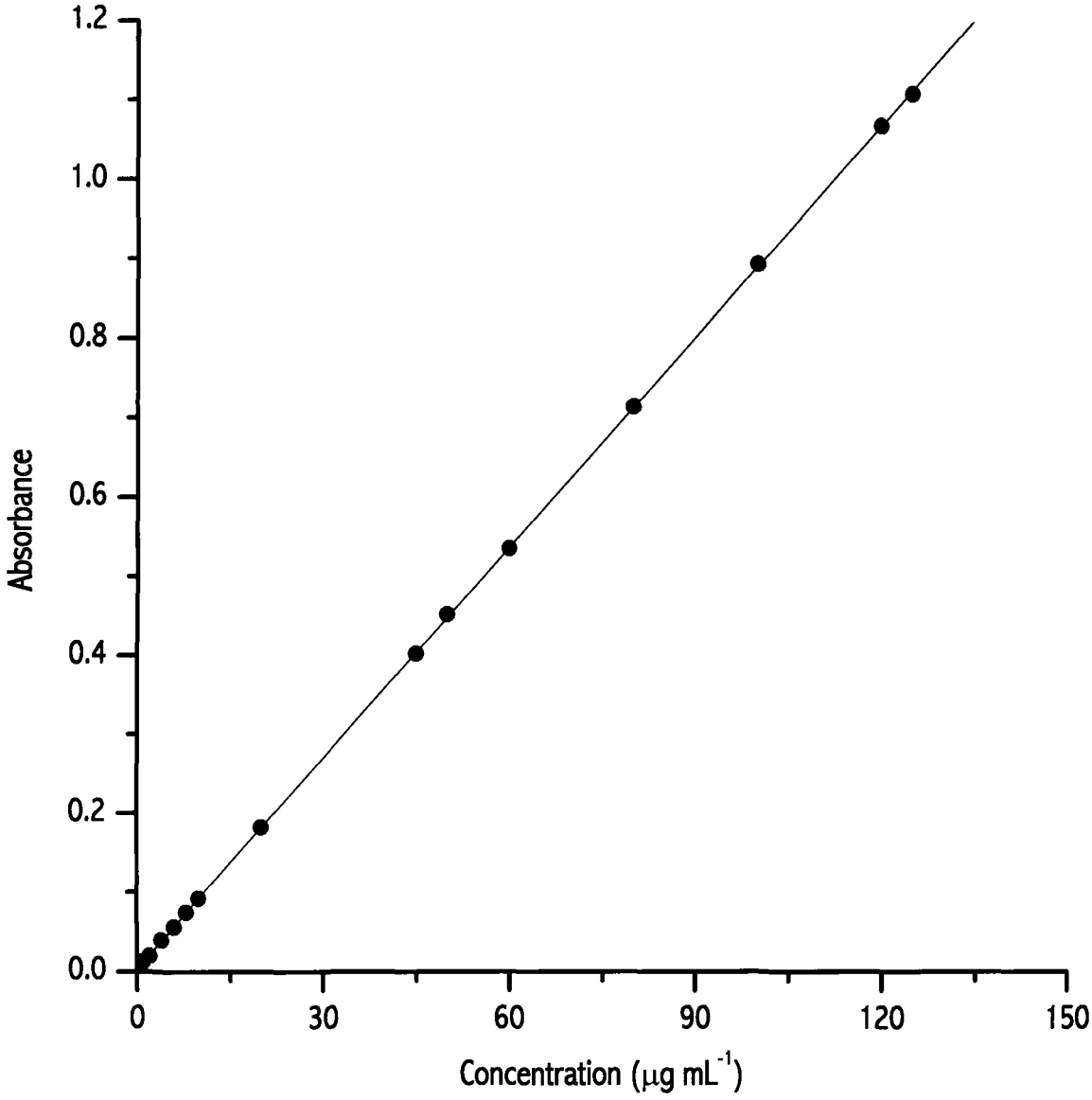


Fig. 4.9. Calibration curve for the determination of amlodipine besylate *(DDQ method).*

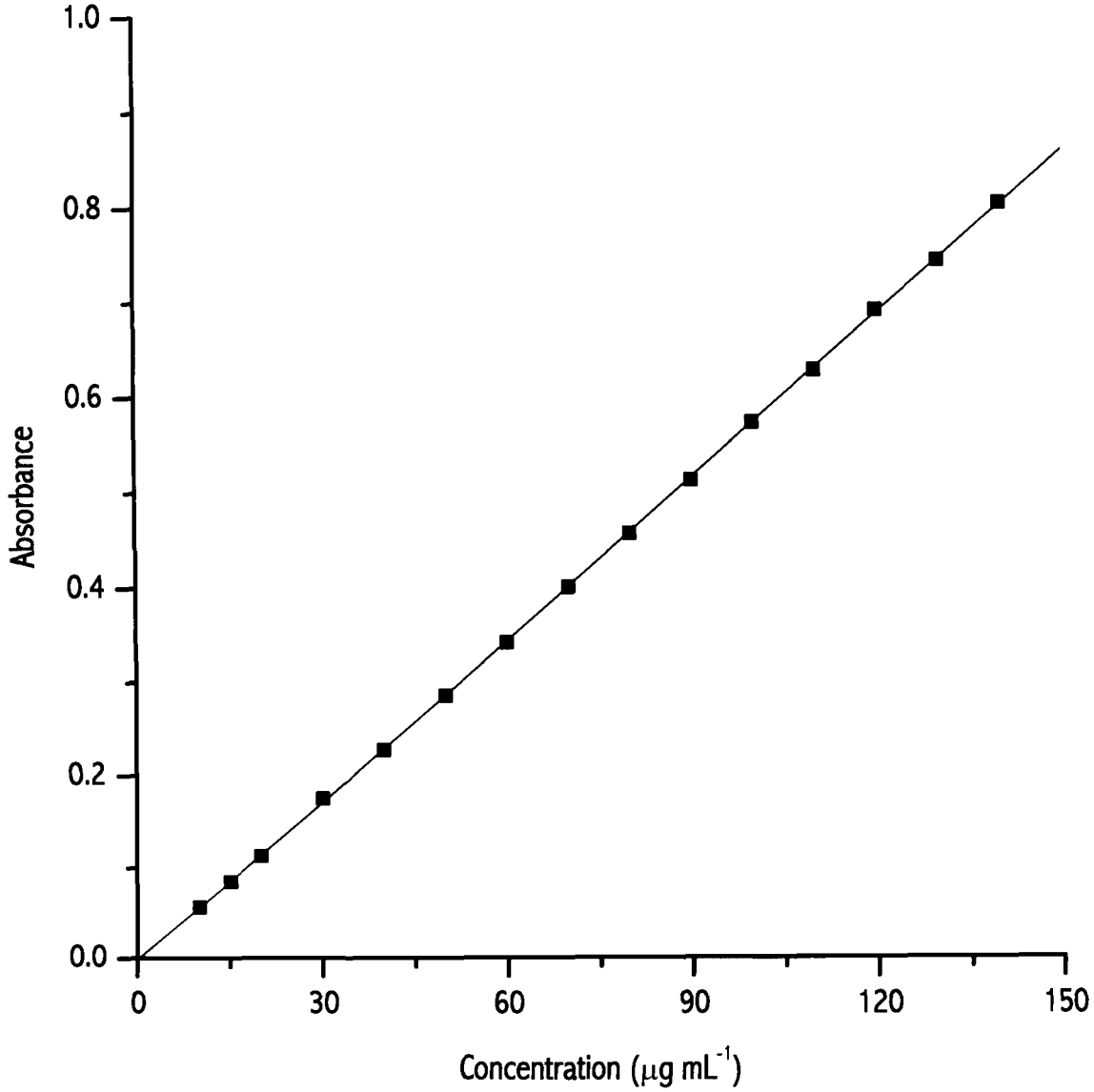


Fig. 4.10. Calibration curve for the determination of amlodipine besylate *(Ascorbic acid method).*

and $A = -1.55 \times 10^{-3} + 5.71 \times 10^{-3} C$, respectively. In each case, the correlation coefficient was found to be 0.9999, indicating the good linearity of both the calibration graphs and the intercepts are all close to zero. The confidence intervals of intercepts at 95% confidence level were calculated (1.45×10^{-3} and 1.90×10^{-3} for DDQ and ascorbic acid methods, respectively) which confirmed that these are not different from zero. Thus the present methods are free from constant errors independent of the concentration of amlodipine besylate. There is also strong correlation existing between the slope and intercept. In order to judge the reliability of strong correlation of these parameters, more rigorous treatment of calibration data was made to draw a joint confidence region (Fig. 4.11 & 4.12), following the method of Mandel and Linnig [46]. The joint confidence region for slope and intercept is a tilt–less ellipse having the point of best fit as its centre. It is evident from Fig. 4.11 and 4.12 that the points for which intercept is zero fall well within the ellipse.

The variance was calculated using the equation $S_o^2 = \Sigma (A_{exp} - A_{calc.})^2 / n - 2$ [47] and found to be 4.44×10^{-6} and 4.50×10^{-5} for DDQ and ascorbic acid methods, respectively. The small values of variance obtained for both the methods indicated negligible scattering of the experimental data points from the line of best fit. The values of correlation coefficients were not sufficient enough to evaluate the linearity of the calibration graphs. The linearity was evaluated by the percent relative standard deviation of the slope ($S_{b\,rel}$ %) [48]. The values were found to be 0.11 and 0.18 for DDQ and ascorbic acid methods, respectively which indicated better linearity of the former method. The statistical analysis of the calibration data also allows the calculation of error (S_{xo}) in the determination of a given concentration of amlodipine besylate and may be helpful to establish the confidence limits at the selected levels of confidence in the

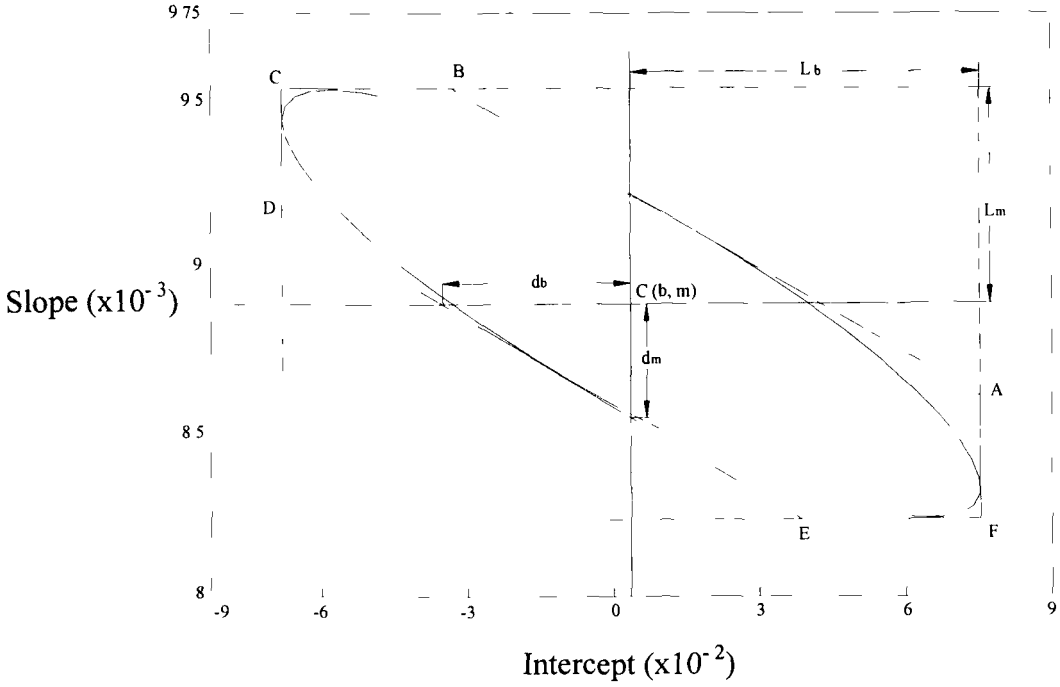


Figure 4.11. Joint confidence region *(at P = 0.05)* for the slope and intercept of the line of regression *(DDQ method).*

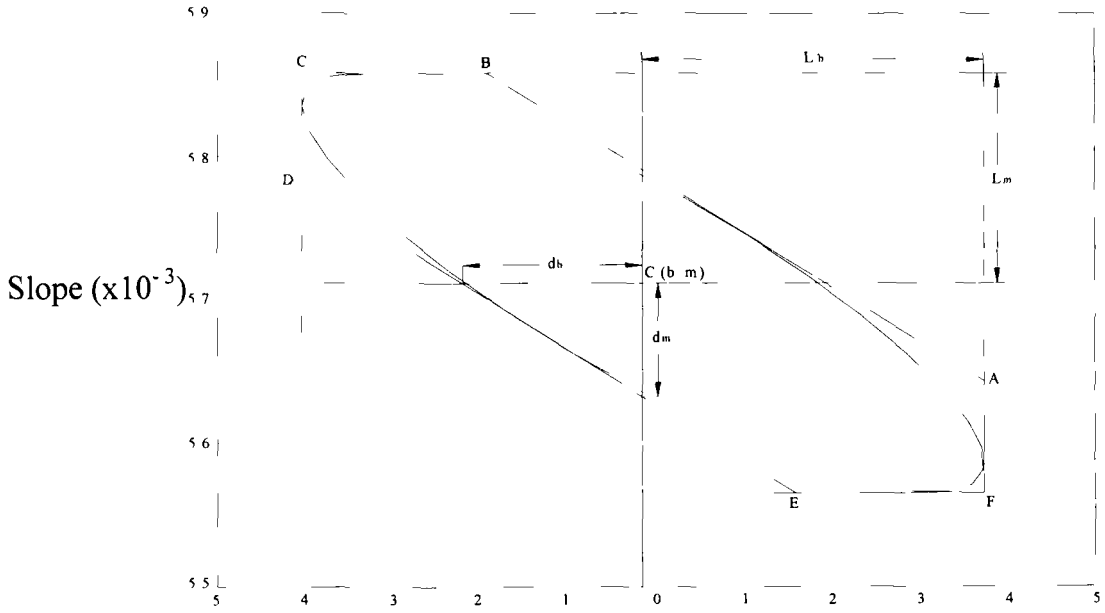


Figure 4.12. Joint confidence region *(at P = 0.05)* for the slope and intercept of the line of regression *(Ascorbic acid method).*

determination of unknown concentrations. These parameters were evaluated by using the following formula [49]. $$S_{x0} = \frac{S_{y/x}}{b}\left[1 + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}\right]^{1/2}$$

Where $\bar{y}$ and $\bar{x}$ are the average absorbance and concentration values, respectively for n standard specimens. Fig. 4.13 and 4.14 shows the graphs of S_{xo} versus concentration of amlodipine besylate. The error is minimum when the actual absorbance is equal to the average absorbance which corresponds to about 45 and 70 μg mL^{-1} for DDQ and ascorbic acid methods, respectively. The accuracy and precision of the proposed methods were evaluated by the repeated analyses at three different concentration levels. The results are summarized in Table 4.1. The standard deviations, relative standard deviations and standard analytical errors [50] can be considered to be very satisfactory.

As an additional demonstration of accuracy, recovery experiments were performed by adding a known amount of amlodipine besylate to the preanalysed dosage forms. The results showed (Table 4.2) that the mean recoveries were in the range of 99.34 – 100.20%. No interference from the common execipients was observed.

The methods were successfully applied to the determination of amlodipine besylate in pharmaceutical formulations. The results of the proposed method (DDQ or ascorbic acid) were compared with those of the reference method [30]. Table 4.3 shows that the calculated *t*–and *F*–values are less than the theoretical ones, confirming accuracy and precision at 95% confidence level. Under the experimental conditions described, the linearity and sensitivity were the best with the charge transfer complex formation procedure. Both the proposed spectrophotometric methods are simple, sensitive and reproducible. Moreover, these procedures are likely to be very suitable for the routine analysis of amlodipine besylate in dosage forms.

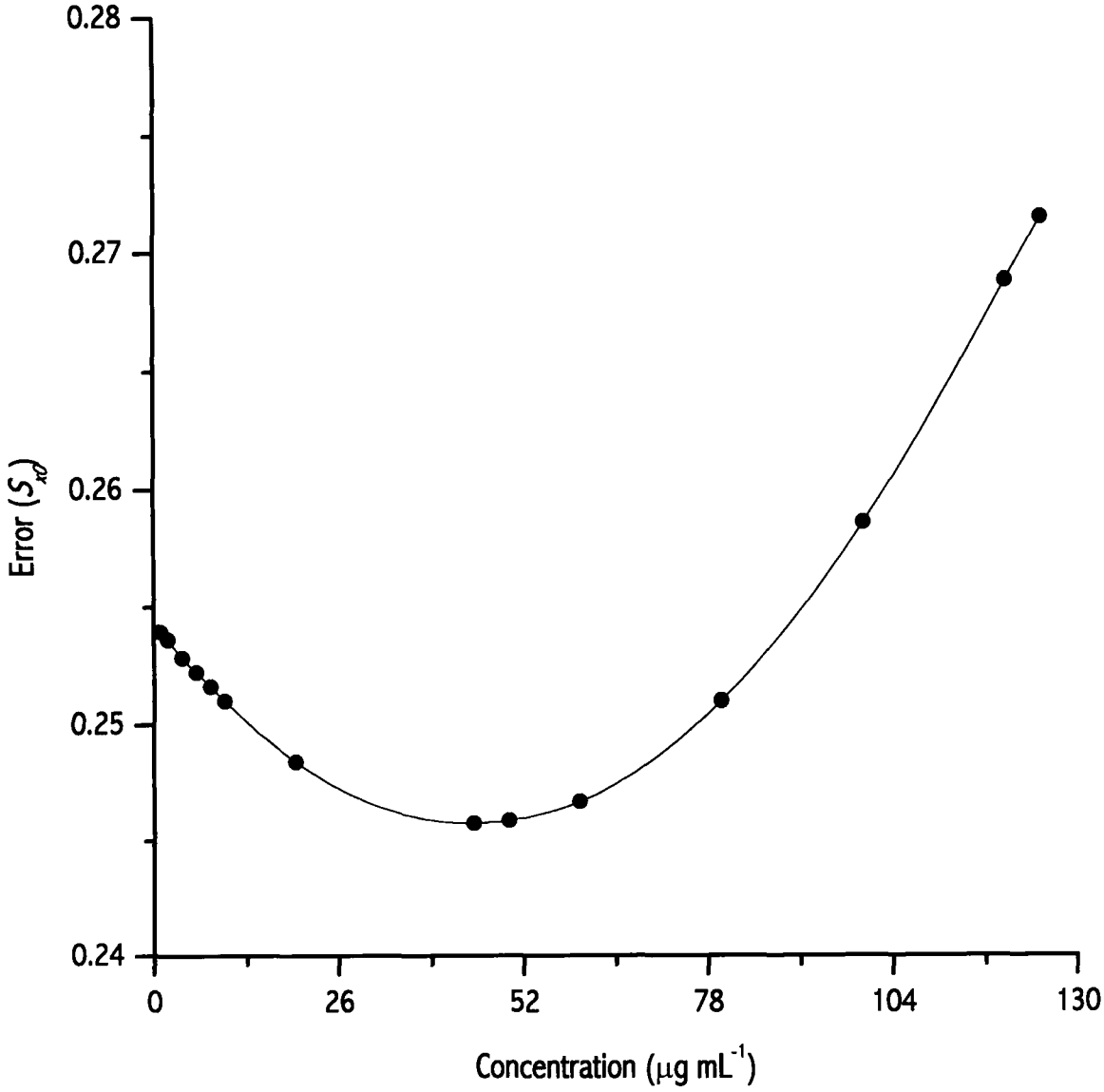


Fig. 4.13. Plot of error in the determination of the concentration of amlodipine besylate by DDQ method.

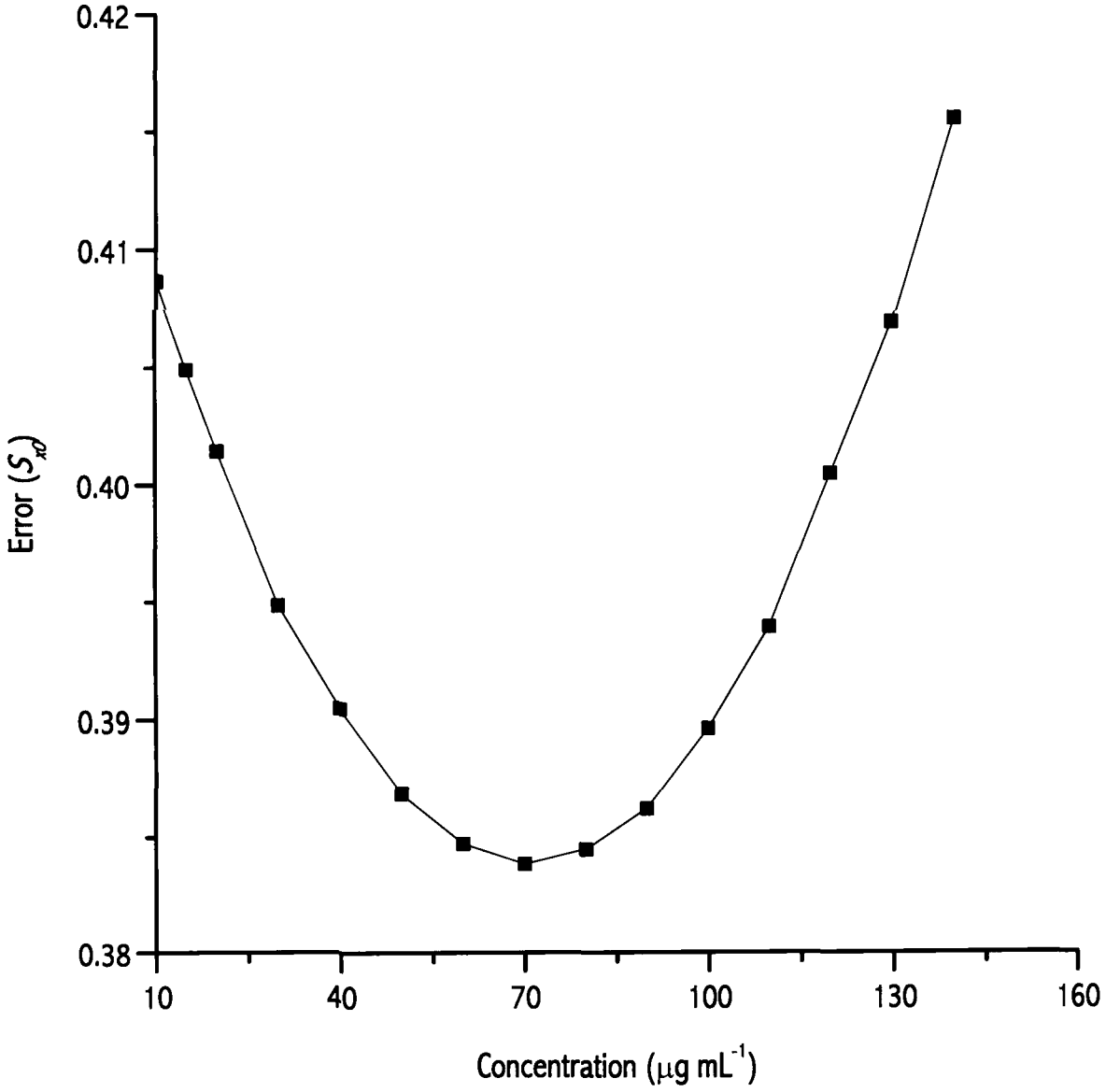


Fig. 4.14. Plot of error in the determination of the concentration of amlodipine besylate by ascorbic acid method.

Table 4.1. Evaluation of the accuracy and precision of the two proposed procedures.

Proposed Methods	Amount taken (mg mL^{-1})	Amount Found (mg mL^{-1}) ± SD[a]	RSD(%)[a]	SAE[b]	Confidence limit[c]
DDQ	10	10 .01 ± 0.06	0.64	0.03	0.061
	60	60.00 ± 0.31	0.51	0.14	0.297
	100	100.03 ± 0.54	0.54	0.24	0.516
Ascorbic acid	20	20.00 ± 0.23	1.14	0.10	0.216
	80	80.03 ± 0.46	0.57	0.20	0.434
	100	100.05 ± 0.51	0.51	0.23	0.490

[a] Mean ± S.D. for five determinations.

[b] SAE, standard analytical error.

[c] Confidence limit at 95% confidence level and 4 degrees of freedom ($t = 2.132$) [51].

Table 4.2. Standard addition method for the determination of amlodipine besylate in dosage forms.

Formulation Name	*DDQ method*						*Ascorbic acid method*					
	Amount (μg mL^{-1})			Recovery (%) ±RSD(%)[a]	*SAE*[b]	Confidence limit[c]	Amount (μg mL^{-1})			Recovery (%) ± RSD(%)[a]	*SAE*[b]	Confidence limit[c]
	taken	added	found ±SD[a]				taken	added	found ± SD[a]			
Amdepin-10	25	25	50 05 ±0 26	100 10 ±0 53	0 12	0 251	15	15	30 03 ±0 29	100 17 ±0 98	0 13	0 280
	35	35	70 01 ±0 32	100 01 ±0 45	0 14	0 303	45	45	89 83 ±0 49	99 81 ±0 55	0 22	0 468
Amlogard 10	25	25	50 06 ±0 25	100 12 ±0 49	0 11	0 234	15	15	30 10 ±0 29	100 33 ±0 95	0 13	0 274
	35	35	70 01 ±0 38	100 03 ±0 54	0 17	0 359	45	45	90 08 ±0 51	100 09 ±0 56	0 23	0 486
Amlong-10	25	25	49 75 ±0 30	99 50 ±0 60	0 13	0 284	15	15	29 96 ±0 31	99 87 ±1 05	0 14	0 299
	35	35	69 54 ±0 34	99 34 ±0 49	0 15	0 328	45	45	89 86 ±0 47	99 84 ±0 52	0 21	0 446
Amlopin-10	25	25	50 03 ±0 29	100 06 ±0 59	0 13	0 281	15	15	30 00 ±0 31	100 01 ±1 02	0 14	0 293
	35	35	69 85 ±0 36	99 79 ±0 52	0 16	0 346	45	45	89 97 ±0 45	99 97 ±0 49	0 20	0 424
Amlopres-10	25	25	49 93 ±0 31	99 86 ±0 62	0 14	0 294	15	15	29 98 ±0 27	99 93 ±0 91	0 12	0 260
	35	35	69 97 ±0 37	99 96 ±1 53	0 17	0 351	45	45	89 91 ±0 48	99 90 ±0 53	0 21	0 453
Myodura	25	25	50 08 ±0 29	100 16 ±0 58	0 13	0 275	15	15	30 01 ±0 28	100 03 ±0 95	0 13	0 271
	35	35	70 14 ±0 33	100 20 ±0 46	0 15	0 312	45	45	90 05 ±0 44	100 05 ±0 49	0 20	0 419

[a] Mean ±SD for five determinations

[b] *SAE*, Standard analytical error

[c] Confidence limit at 95% confidence level and four degrees of freedom (t = 2 132) [51]

Table 4.3. Comparison of the two proposed methods with the reference method [30].

Pharmaceutical preparations	Labelled amount (mg)	DDQ Method				Ascorbic Acid Method				Reference Method	
		Recovery (%)[a]	RSD (%)[a]	*t*– value[b]	*F*– value[b]	Recovery (%)[a]	RSD (%)[a]	*t*– value[b]	*F*– value[b]	Recovery (%)[a]	RSD (%)[a]
Amdepin	10	100.13	0.73	0.0404	4.51	99.90	0.59	0.7370	2.93	100.11	0.34
Amlogard	10	100.20	0.67	0.2952	4.20	99.98	0.57	0.3664	2.98	100.09	0.33
Amlong	10	99.95	0.32	0.0614	1.85	99.98	0.36	0.0351	2.41	99.98	0.24
Amlopin	10	99.96	0.38	0.0434	1.11	99.72	0.84	0.6133	4.46	99.97	0.40
Amlopres	10	99.98	0.51	0.5369	1.22	99.75	0.95	0.8667	2.81	100.14	0.55
Myodura	10	100.19	0.68	0.4451	2.72	99.80	0.67	0.6463	2.60	100.03	0.41

[a] Average of five independent analyses.

[b] Theoretical *t*–value ($DOF = 8$) and *F*–value ($DOF = 4,4$) at 95% confidence level are 1.860 and 6.39, respectively [51].

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Chapter - 5

Kinetic spectrophotometric methods for the determination of diltiazem hydrochloride in pharmaceutical formulations

Introduction

Diltiazem hydrochloride is an important coronary vasodilator, which reduces total peripheral vascular resistance and is included in the calcium channel–blocking group of the antianginal drugs. It is chemically (+)–5–(p–methoxyphenyl)–1,5–benzothiazepin–4(5H)–one acetate monohydrochloride, and its tablet formulations are listed in the United States, British and Indian pharmacopoeias [1–3]. The drug is used in the treatment of angina and hypertension [4] and also exhibits direct but weak negative ionotropic and chronotropic effects [5–7]. Due to its characteristic short half-life ($t_{1/2}$) diltiazem requires frequent dosing in therapeutic situations.

The officially listed methods in the pharmacopoeias for the determination of diltiazem are based on the high performance liquid chromatography [1,3] and titrimetry [2]. Few other high performance liquid chromatographic methods have also been proposed in the literature [8–14]. Certain other analytical techniques such as gas chromatography [15,16], high performance thin layer chromatography [17], capillary electrophoresis [18] and titrimetry [19] were also utilised for its quantification. Based on the inherent property of optical rotation in the diltiazem molecule, Li and Qui have developed a polarimetric method for its determination [20]. In the literature few spectrophotometric methods have also been reported. These methods are based on the formation of ferric hydroxamate due to acetate moiety in the diltiazem molecule [21,22], oxidation of the drug with Fe(III) or excess of N–bromosuccinimide and the determination of subsequently produced Fe(II) with 1,10–phenanthroline or unconsumed N–bromosuccinimide with metol–sulfanilamide [23], reaction of hydrolysed diltiazem hydrochloride with Folin–Ciocalteau reagent [24]. Some other spectrophotometric

methods have also been developed which involved the charge transfer reactions [25], oxidation in strong sulphuric acid medium [26] and extractable ion-pair complexes of the drug [27–30].

To the best of our knowledge, no kinetic spectrophotometric method has been proposed for the assay of diltiazem hydrochloride. Potassium permanganate is an economical and versatile oxidising agent, which has been frequently used in the kinetic spectrophotometric determination of pharmaceuticals in different media [31–33]. In this study diltiazem hydrochloride undergoes oxidation with potassium permanganate in alkaline medium and the change in absorbance was measured as a function of time at 610 nm and 530 nm. The main aim of this work was, therefore, to study the kinetic data and evaluate their analytical applicability for the assay of drug in pharmaceutical formulations.

Experimental

Instrument

The absorbance was recorded on a Spectronic 20D$^+$ spectrophotometer (Milton Roy, USA) with matched glass cuvettes.

Materials

All the chemicals used were of analytical reagent grade. Diltiazem hydrochloride was obtained from Sigma Chemical Co. (USA) and was used as received. The commercial diltiazem hydrochloride tablets were purchased locally.

Reagents and solutions

Stock solution of diltiazem hydrochloride (1 mg mL^{-1}) was prepared in doubly distilled water and it was further diluted according to the need. Potassium permanganate (2.5 mg mL^{-1}) and 1 M sodium hydroxide solutions were also prepared in doubly distilled water.

General procedure for the determination of diltiazem hydrochloride

(1) *Formation kinetics method*

1 mL of potassium permanganate (12.66 $x10^{-3}$ M) and 1 mL of sodium hydroxide (1 M) were pipetted into a series of 10 mL volumetric flasks. Then 1 mL of diltiazem hydrochloride of varying concentrations (1 – 7 µg mL^{-1}) was added to each flask and diluted to volume with doubly distilled water. The content was mixed well and immediately transferred to cuvette. The absorbance at 610 nm was measured as a function of time against the reagent blank (Fig. 5.1). The intercept and slope from the initial straight line of the absorbance–time curve was obtained. The calibration graphs were constructed by plotting log of intercept vs. the log of molar concentration of the drug (log C), log of slope vs. log C and absorbance measured at 610 nm at a fixed time of 14 minutes vs. the concentration of the drug.

(2) *Degradation kinetics method*

1 mL aliquots of potassium permanganate (5.06 $x10^{-3}$), 1 mL of 1 M sodium hydrochloride and varying concentrations of diltiazem hydrochloride (4 – 12 µg mL^{-1}) were transferred into a series of 10 mL volumetric flasks and diluted to the mark with distilled water. The decrease in the absorbance at 530 nm was recorded as a function of time against the distilled water (Fig. 5.2). The initial rate of reaction (ν) was obtained from the

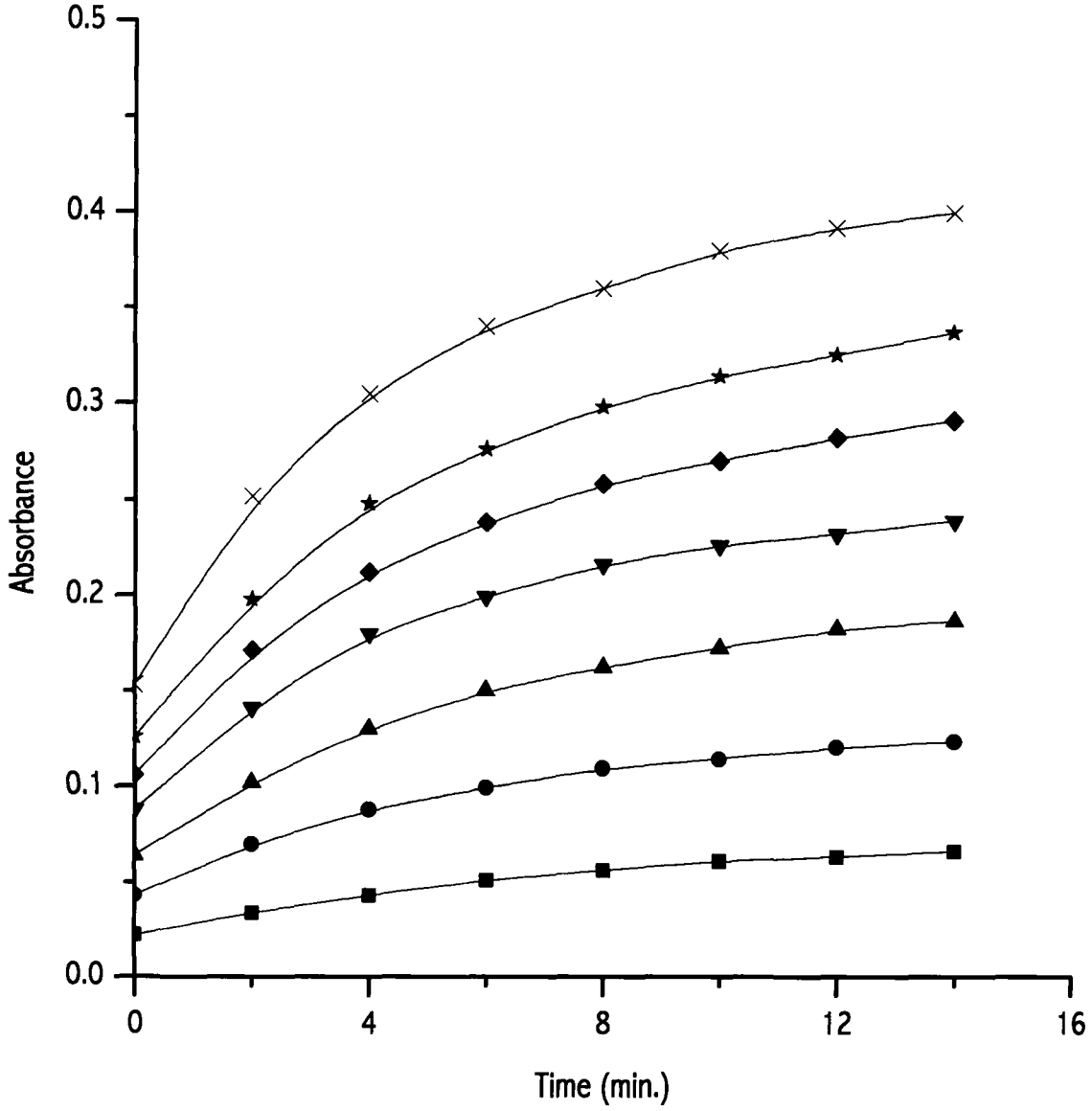


Fig. 5.1. Absorbance–time curve for the varying concentrations of diltiazem hydrochloride and fixed potassium permanganate concentration (12.66 x10^{-3} M). (■) 2.22 x10^{-6} M; (●) 4.43 x10^{-6} M; (▲) 6.65 x10^{-6} M; (▼) 8.87 x10^{-6} M; (◆) 11.09 x10^{-6} M; (★) 13.30 x10^{-6} M; (×) 15.52 x10^{-6} M.

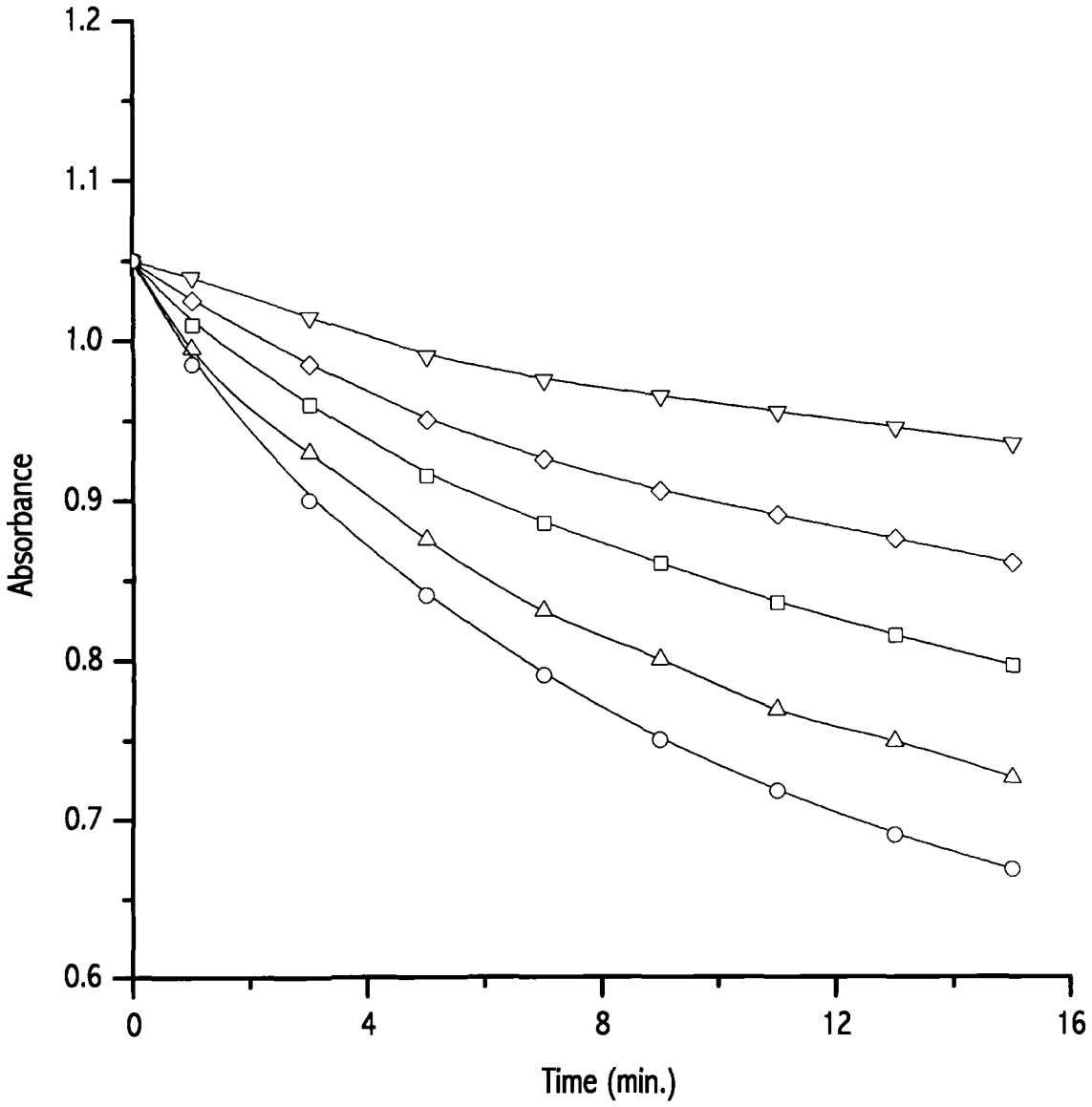


Fig. 5.2. Absorbance–time curve for the varying concentrations of diltiazem hydrochloride and fixed concentration of potassium permanganate (5.06 $x10^{-3}$ M). (○) 8.87 $x10^{-6}$ M; (Δ) 13.30 $x10^{-6}$ M; (□) 17.74 $x10^{-6}$ M; (◇) 22.17 $x10^{-6}$ M; (▽) 26.61 $x10^{-6}$ M.

slope of the tangent to the absorbance–time curve. The calibration curve was prepared by plotting the log of the initial rate of reaction (log ν) vs. log C. The amount of drug is computed either from the calibration curves or regression equations.

Analysis of pharmaceutical preparations

10 tablets of diltiazem hydrochloride (30 mg of diltiazem hydrochloride per tablet) were finely powdered and dissolved in water. Diltiazem hydrochloride content was extracted into chloroform. The organic layer was passed through the anhydrous sodium sulphate and collected with its washings in a beaker, which was evaporated to dryness. The residue was dissolved in water and diluted according to the need. The content of diltiazem was determined following the recommended procedures.

Results and discussion

It is well known that potassium permanganate behaves as a strong oxidant. The alkaline potassium permanganate oxidises diltiazem hydrochloride producing green colour owing to the formation of manganate ion, which absorbed maximally at 610 nm. The intensity of green colour increases with time and hence, this property has been exploited to develop a kinetically based method for the determination of diltiazem hydrochloride. It has also been observed that the intensity of purple colour (λ_{max} 530 nm) decreases with time and therefore, another method based on the degradation kinetics was also elaborated. In order to come to this conclusion, some preliminary experiments were performed to optimise the temperature, reagent concentration and alkalinity.

Effect of temperature

The reaction between diltiazem hydrochloride and alkaline potassium permanganate proceeds at room temperature with substantial change in the absorbance with time. The initial rate of reaction increases with increase in temperature, resulting an increase in the slope of the calibration graph. This suggested a higher analytical sensitivity but at the same time a significant decrease in the linearity was noticed which might be due to unwanted chemical changes. Therefore, all the absorbance measurements were made at room temperature throughout the experiment.

Effect of potassium permanganate concentration

The effect of 1 mL of varying concentrations of potassium permanganate (1.27×10^{-3} – 13.92×10^{-3} M) on the reaction rate at 610 nm was studied by a fixed time method (14 minutes) and was found to be constant above 11.39×10^{-3} M (Fig. 5.3). Therefore, 1 mL of 12.66×10^{-3} M $KMnO_4$ solution was used throughout the experiment carried at 610 nm. The influence of reagent's concentration on the initial rate of reaction at 530 nm was also examined. The initial rate of reaction increases with increasing concentration of potassium permanganate and became constant after 4.43×10^{-3} M. So finally 5.06×10^{-3} M $KMnO_4$ solution was taken as the optimum concentration in the degradation kinetics method (Fig. 5.4).

Effect of sodium hydroxide concentration

The effect of sodium hydroxide concentration was investigated in the similar manner as for $KMnO_4$. The results in both, the formation as well as the degradation methods, show that 1 mL aliquot of sodium hydroxide has no effect either on the absorbance or the rates above 0.9 M (Fig. 5.5).

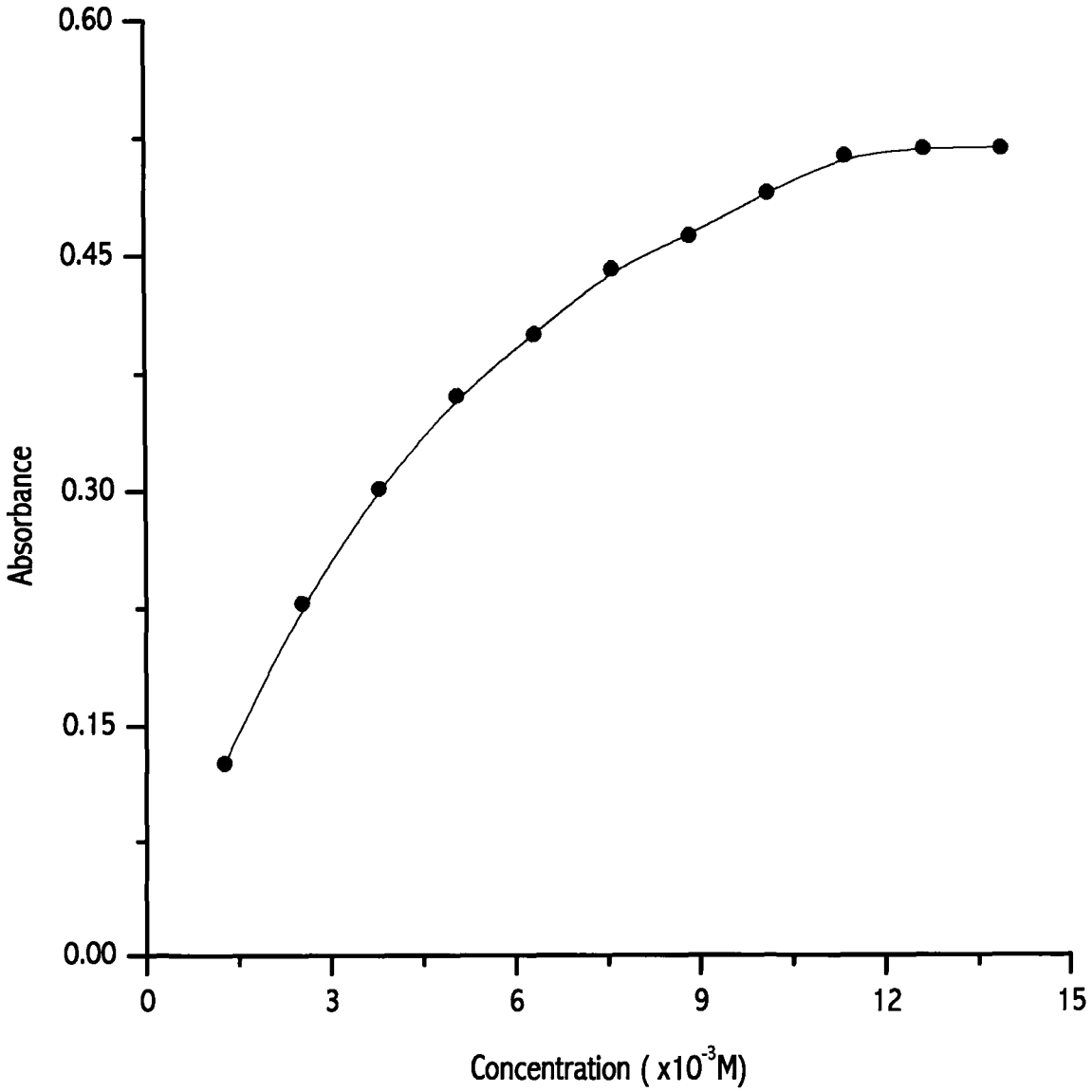


Fig. 5.3. Effect of 1 mL of varying concentrations of potassium permanganate.

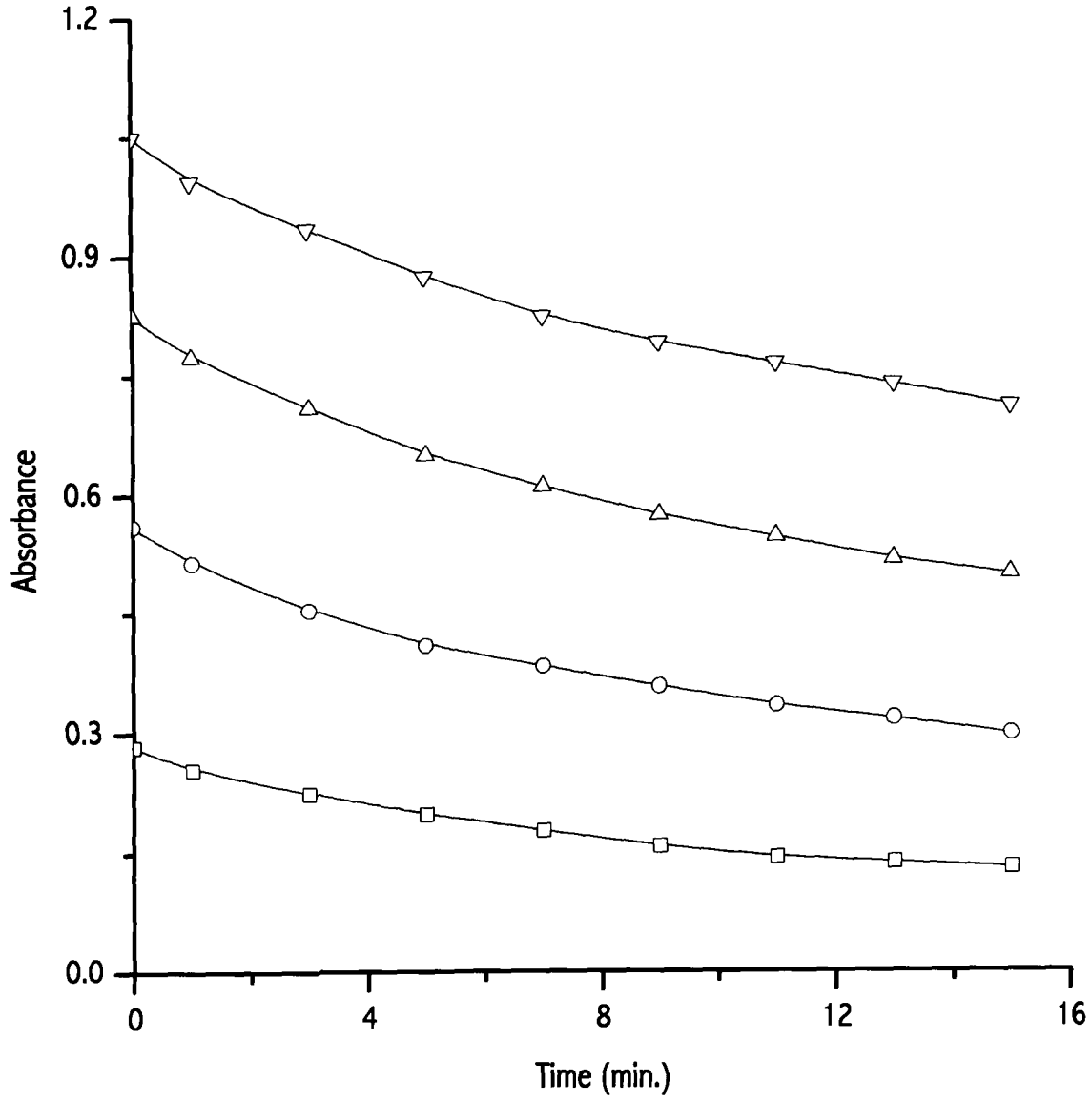


Fig. 5.4. Absorbance–time curve for the varying concentrations of potassium permanganate and fixed diltiazem hydrochloride concentration (2.22×10^{-5} M). (□) 1.27×10^{-3} M; (○) 2.53×10^{-3} M; (△) 3.80×10^{-3} M; (▽) 5.06×10^{-3} M.

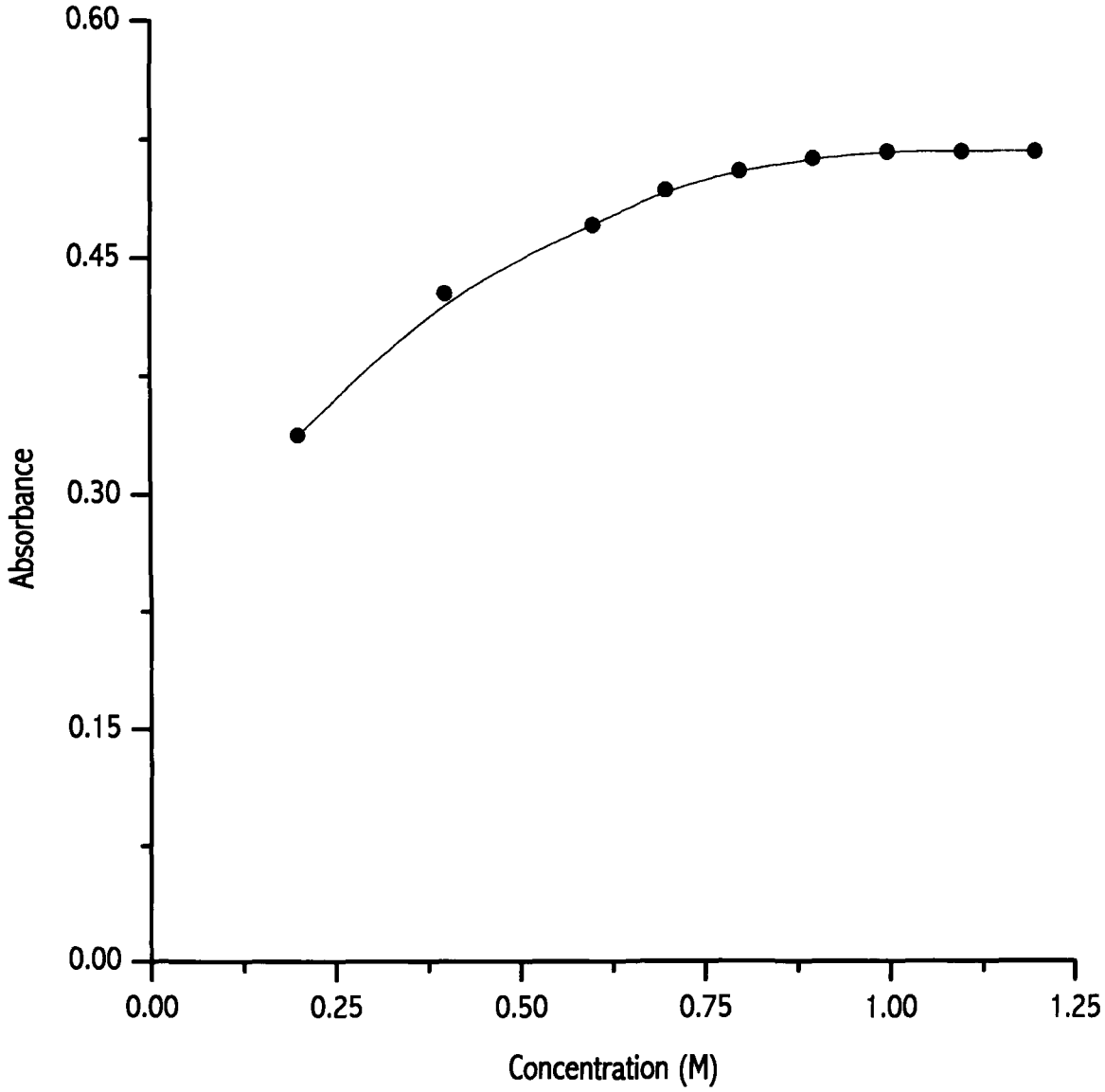


Fig. 5.5. Effect of 1 mL of varying concentrations of sodium hydroxide.

Stoichiometry and mechanism of the reaction

In order to establish the molar ratio between potassium permanganate and diltiazem hydrochloride, the limiting logarithmic method [34] was employed. For this purpose, two sets of experiments were performed; one with constant $KMnO_4$ concentration and varying diltiazem hydrochloride concentration (Fig. 5.1) and another with constant diltiazem hydrochloride concentration and varying $KMnO_4$ concentration (Fig. 5.6). The logarithms of absorbances of the two sets were plotted against the logarithms of the respective varied concentrations (Fig. 5.7). The slope of the two curves gave the number of moles of diltiazem hydrochloride and $KMnO_4$, respectively. The molar ratio was found to be 1 : 1 for diltiazem hydrochloride/potassium permanganate.

The diltiazem molecule possesses a thioether linkage, which is susceptible to oxidation to the corresponding sulphoxide. On the basis of the experimental findings and literature background, a tentative reaction mechanism is proposed and given in Scheme 5.1.

Statistical analysis and analytical applicability

Under the optimised experimental conditions, calibration graphs were constructed. The plots of **(i)** log intercept vs. log C (Fig. 5.8); **(ii)** log initial rate of formation of MnO_4^{-2} vs. log C (Fig. 5.9); **(iii)** absorbance (610 nm) measured at a fixed time of 14 minutes vs. concentration (Fig. 5.10); and **(iv)** log rate of degradation of potassium permanganate (530 nm) vs. log C (Fig. 5.11), showed a linear dynamic range of 1 – 7, 1 – 5, 1 – 7 and 4 – 12 μg mL^{-1}, respectively. In view of the extremely sensitive nature of the reaction and narrow linear dynamic range, rigorous statistical linearity tests were performed with repeated measurements.

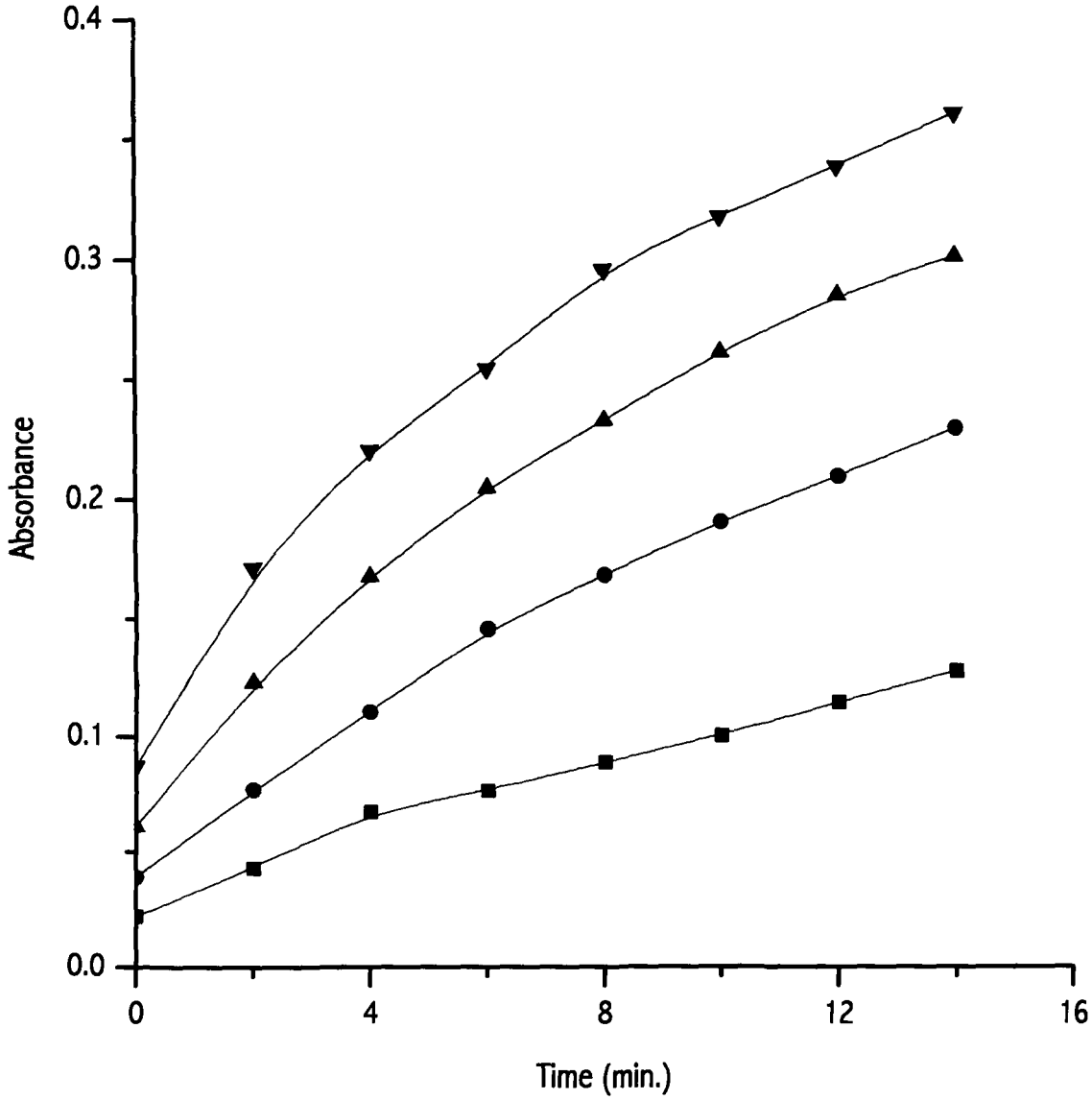


Fig. 5.6. Absorbance–time curve for the varying concentrations of potassium permanganate and fixed diltiazem hydrochloride concentration (2.22×10^{-5} M). (■) 1.27×10^{-3} M; (●) 2.53×10^{-3} M; (▲) 3.79×10^{-3} M; (▼) 5.05×10^{-3} M.

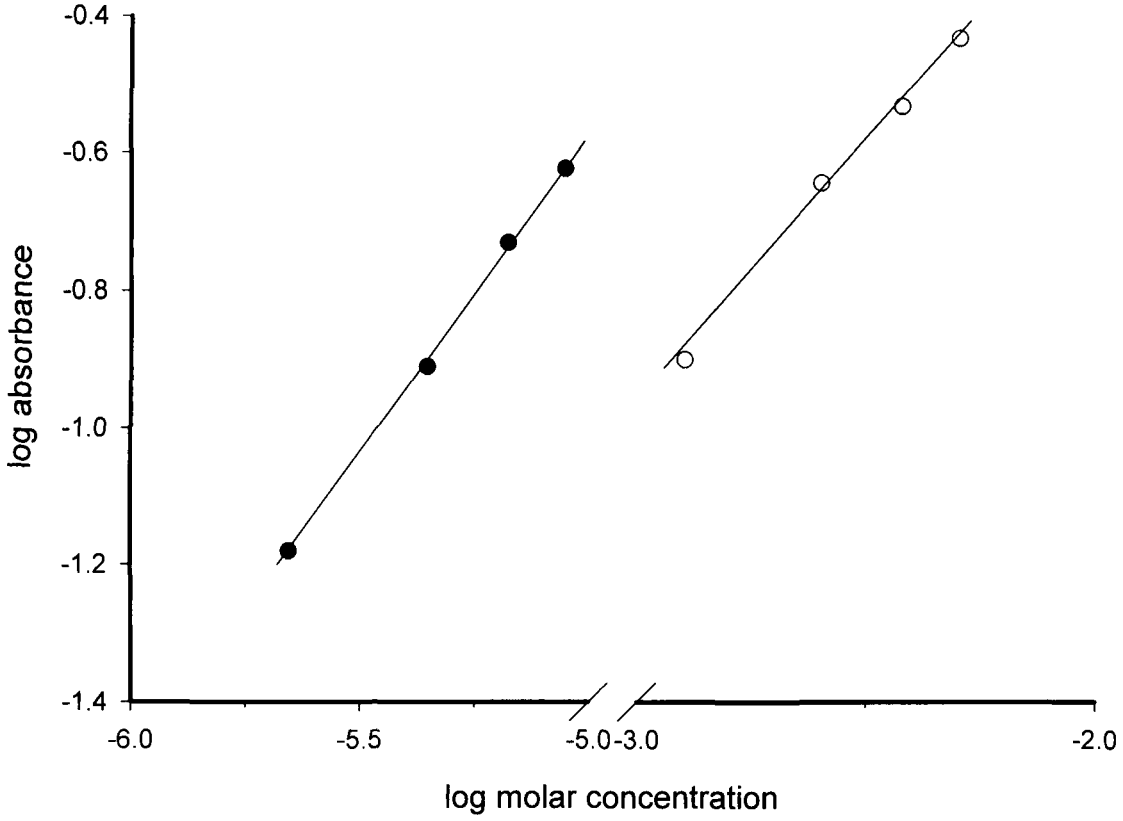
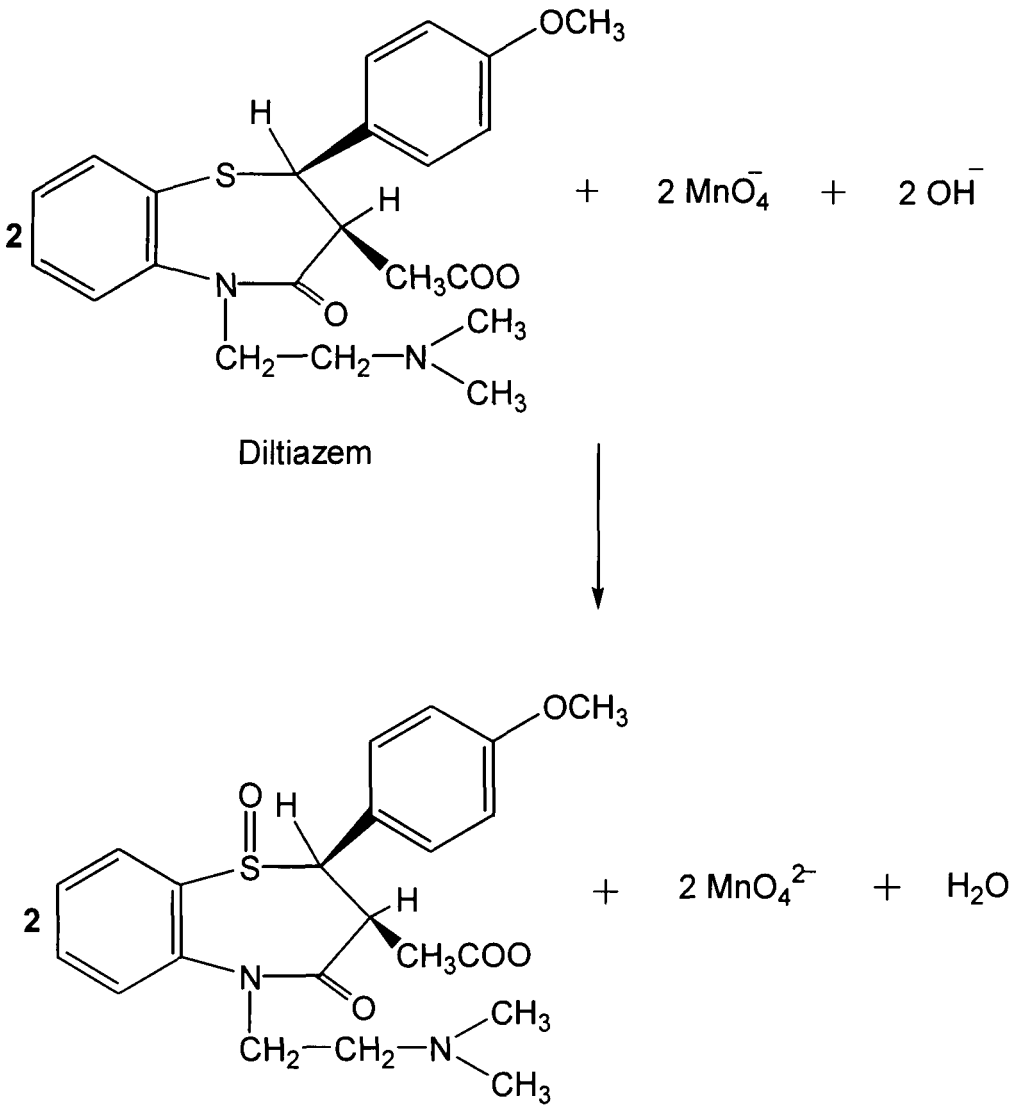


Fig. 5.7. Determination of the molar ratio between diltiazem hydrochloride and potassium permanganate by limiting logarithmic method. Set of solutions with: (●) constant potassium permanganate concentration and variable diltiazem hydrochloride concentrations, and (○) constant diltiazem hydrochloride concentration and variable potassium permanganate concentrations.



Sulphoxide derivative of diltiazem

SCHEME 5.1

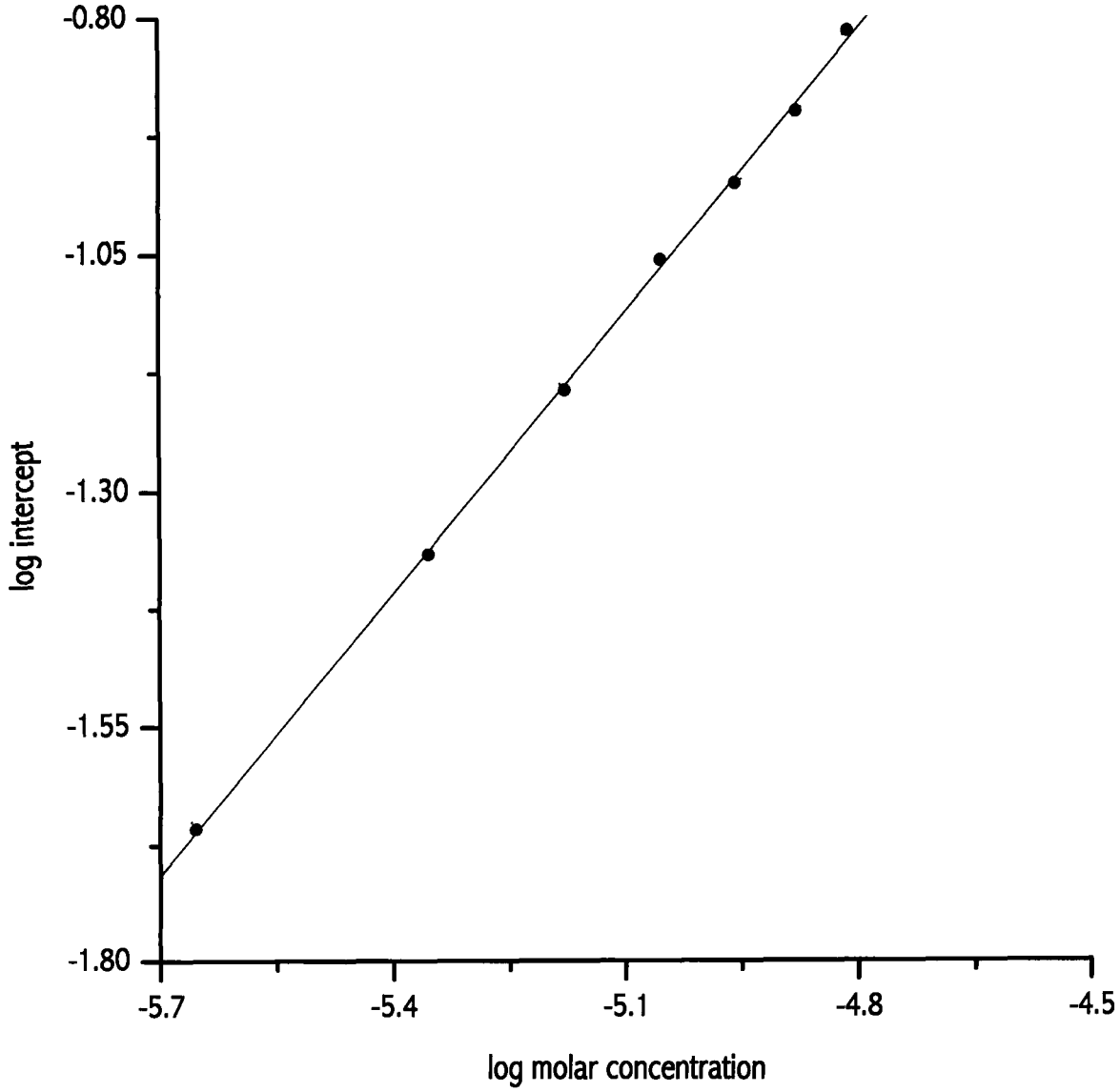


Fig. 5.8. Calibration curve *(formation kinetics method):* plot of log intercept vs. log molar concentration of diltiazem hydrochloride (——) and 95% confidence band (.....).

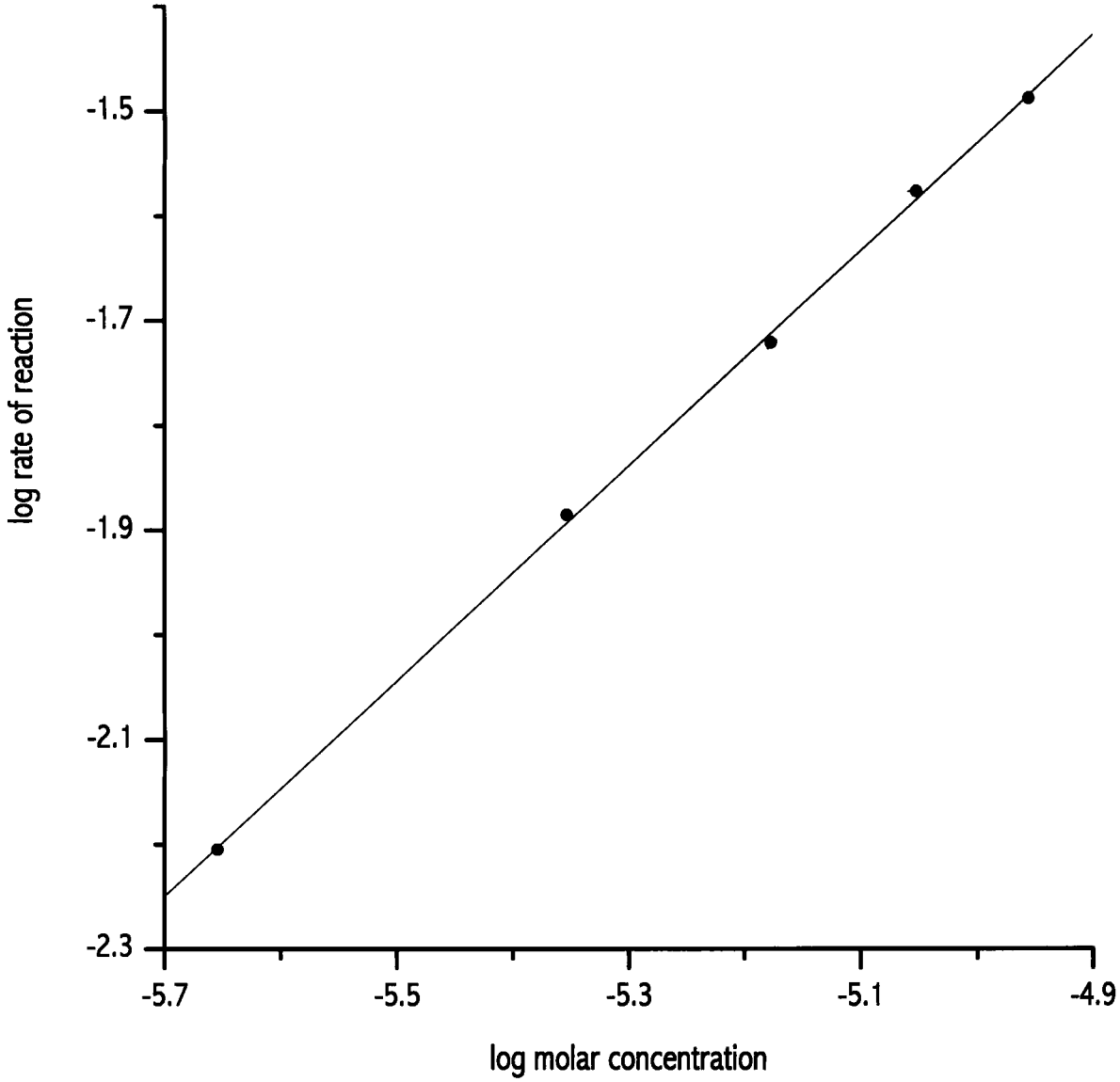


Fig. 5.9. Calibration curve *(formation kinetics method):* plot of log initial rate of reaction vs. log molar concentration of diltiazem hydrochloride (—-) and 95% confidence band (....).

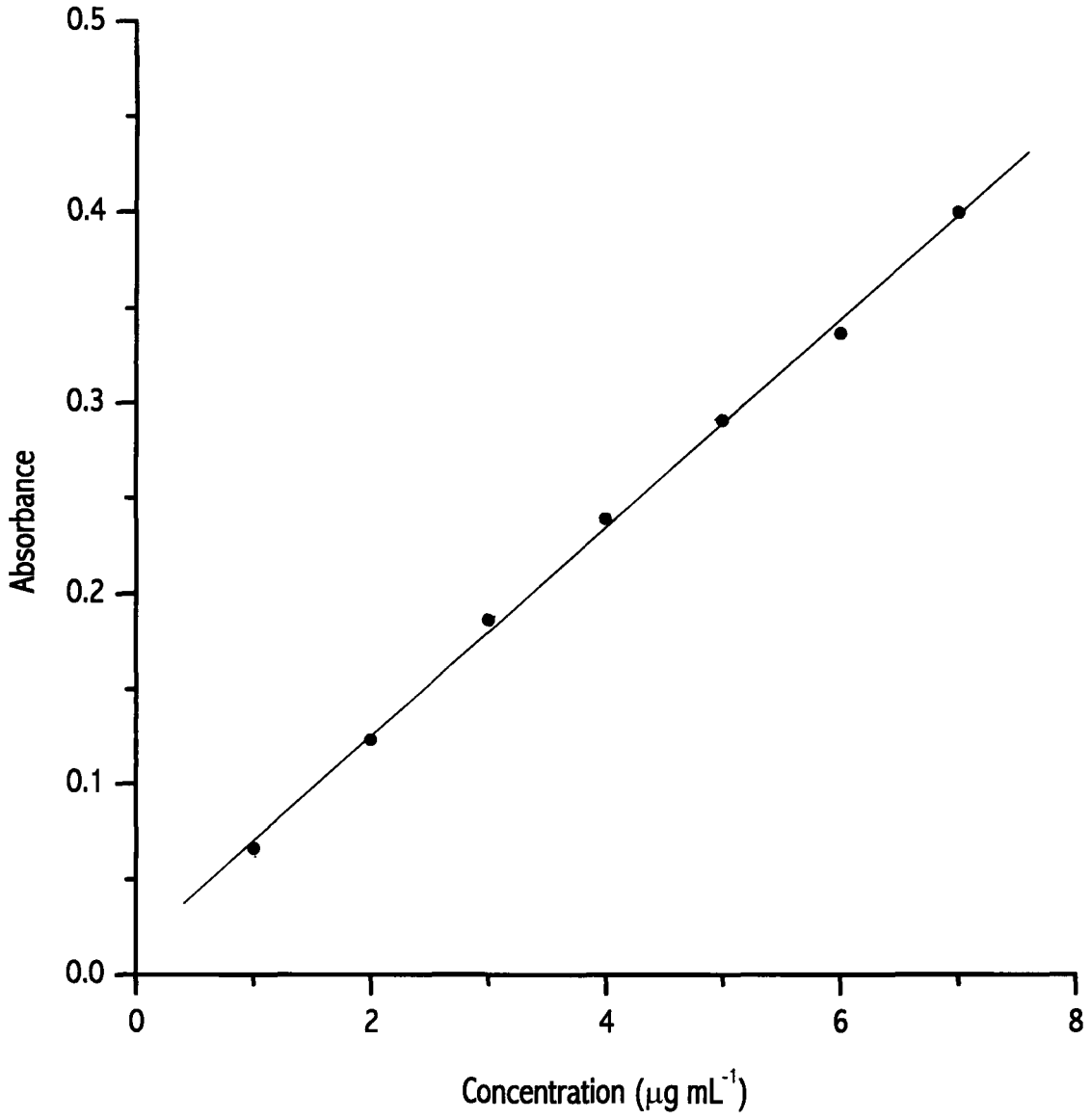


Fig. 5.10. Calibration curve *(formation kinetics method):* plot of absorbance (610 nm) measured at a fixed time of 14 minutes vs. concentration of diltiazem hydrochloride (—-) and 95% confidence band (....).

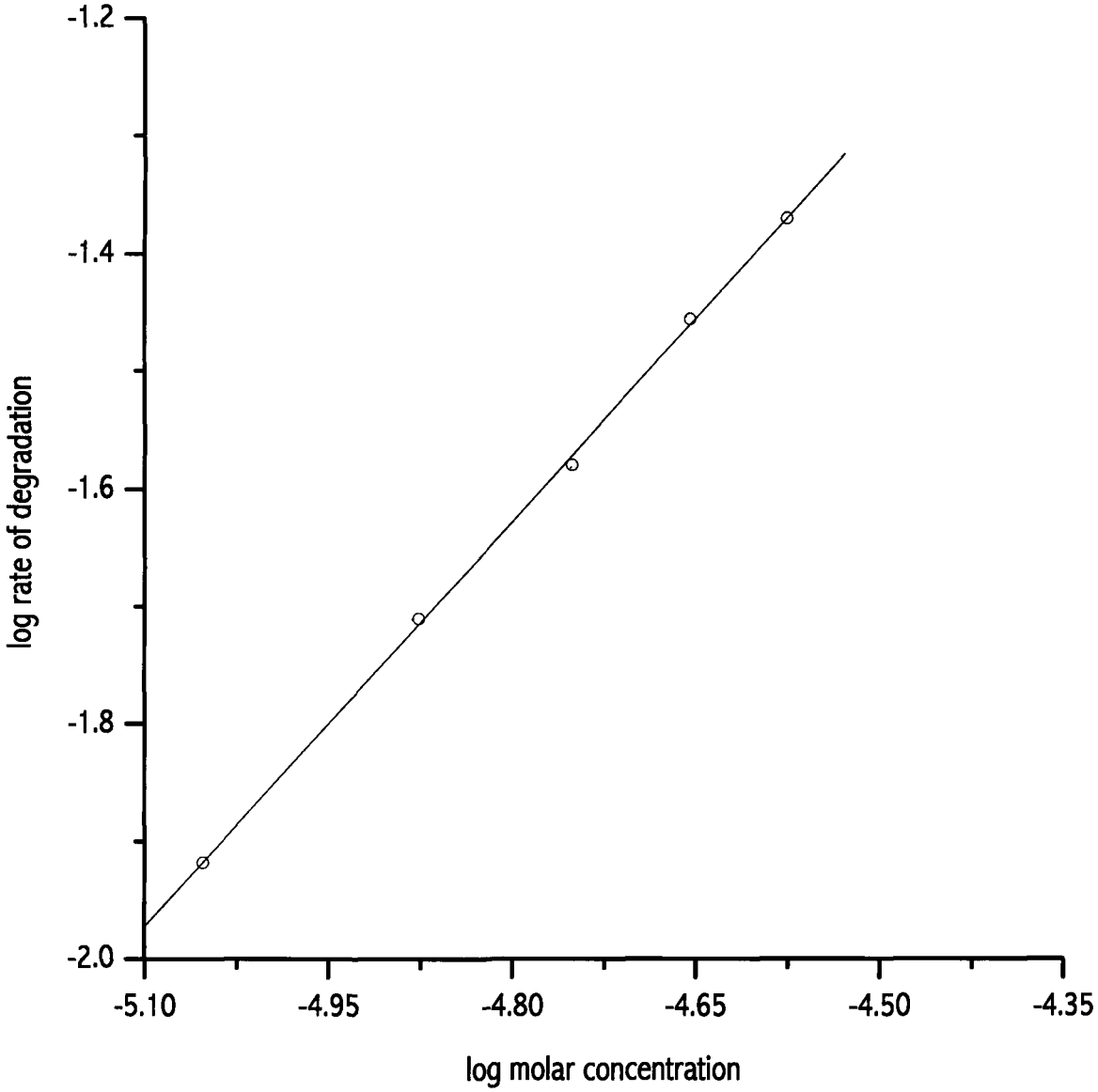


Fig. 5.11. Calibration curve *(degradation kinetics method):* plot of log initial rate of degradation vs. log molar concentration of diltiazem hydrochloride (——) and 95% confidence band (.....).

The main figures of merit are presented in Table 5.1 and 5.2. In all the cases, the distributions were found to be normal as indicated by the David's test [35, 39] wherease the corresponding variances were homoscedastic when Cochran test [36, 39] was applied. The test for outlier among the calibration data was performed using Dixon test and Grubb's single and double outlier tests [37–39]. The results show that there is no outlier among the set of data used for the calibration purpose. The significance of linear regression method was established by the comparative study with the quadratic regression method. In all the cases, the residual variance of the linear regression was found to be less than that of the quadratic regression (Table 5.2). This clearly indicates a significantly better fit by the linear regression. It was again favoured by the Mandel test in which the calculated values were found to be less than the critical values. Moreover, the correlation coefficient *(r)* has been often used as a crude parameter for linearity on the ground that a linear calibration function usually gives a high correlation coefficient and thus misleading in the context of testing for linearity. So, in fact, a numerical value of r can not be interpreted in terms of degree of deviation from linearity. Therefore, to assure the goodness of fit in the calibration graphs, lack of fit test was performed. The results obtained are summarised in Table 5.3. It is evident from the data obtained that the F– value found by the ratio of the mean of squares of the lack of fit value to that of the pure error is less than the tabulated values; thus indicating that the goodness of fit is maintained in all the sets of the calibration data.

For the evaluation of precision estimates, repeatability and intermediate precision were performed at three different concentration levels throughout the calibration range. The results obtained are summarised in Table 5.4. In all the cases, intraserial variances were found to be less than their corresponding interserial variances, thus it does not require to perform the

Table 5.1. Regression parameters of the different calibration methods.

Methods	Linear dynamic range (μg mL^{-1})	Regression equation	Standard error of estimate	Limit of	
				Detection (μg mL^{-1})	Quantitation (μg mL^{-1})
Formation methods					
Intercept method	1 – 7	*log Intercept= 3.938 + 0.9903 log C* (*r = 0.9997*)	8.08×10^{-3}	2.64×10^{-2}	8.00×10^{-2}
Rate of formation method	1 – 5	*log ν = 3.606 + 1.0272 log C* (*r = 0.9997*)	8.07×10^{-3}	2.59×10^{-2}	7.84×10^{-3}
Fixed time					
(14 minutes)	1 – 7	$A = (1.53 + 5.48\ C) \times 10^{-2}$ (*r = 0.9992*)	5.19×10^{-3}	3.13×10^{-1}	9.47×10^{-1}
Degradation method					
Rate of degradation method	4 – 12	*log ν = 3.881+ 1.1476 log C* (*r = 0.9997*)	6.20×10^{-3}	1.10×10^{-2}	3.34×10^{-2}

Table 5.2. Statistical linearity test and significance of linear and quadratic coefficients [39].

Methods	Test for outliers		David's test†	Cochran test†	Significance of quadratic coefficient			
	Dixon	Grubb			Residual variance ($x10^{-5}$)		Mandel test‡	Confidence interval
					Linear	Quadratic		
Formation kinetics								
Intercept method	None	None	Normal	Homoscedasticity	6.52	7.23	0.51 (7.71)	$(-0.96 - 1.62) x10^{-1}$
Rate of formation method	None	None	Normal	Homoscedasticity	6.49	9.07	0.15 (18.15)	$(-3.93 - 3.31) x10^{-3}$
Fixed time								
(14 minutes)	None	None	Normal	Homoscedasticity	2.40	2.70	1.61 (7.71)	$(-2.16 - 0.81) x10^{-3}$
Degradation kinetics								
Rate of degradation method	None	None	Normal	Homoscedasticity	1.47	1.83	0.42 (18.51)	$(-3.89 - 3.03) x10^{-1}$

† For two repeated measurements.

‡ Values in the parentheses are critical [39].

Table 5.3. Analysis of variance with lack of fit.

Methods	Sum of squares	D_f*	Mean squares	F–ratio
log intercept				
Residual	0.0003281	12	0.0000273	
Lack of fit	0.0002071	5	0.0000414	2.394
Pure Error	0.0001210	7	0.0000173	
log rate of formation				
Residual	0.0001508	8	0.0000189	
Lack of fit	0.0001070	3	0.0000357	4.057
Pure error	0.0000438	5	0.0000088	
Fixed time				
(14 minutes)				
Residual	0.0001593	12	0.0000133	
Lack of fit	0.0001003	5	0.0000201	2.393
Pure error	0.0000590	7	0.0000084	
log rate of degradation				
Residual	0.0002068	8	0.0000259	
Lack of fit	0.0001186	3	0.0000395	2.244
Pure error	0.0000882	5	0.0000176	

* Degrees of freedom.

Table 5.4. Analysis of variance of the precision studies with repeatability and intermediate precision [39].

Parameters \ Methods → ↓		Formation kinetics methods									Degradation kinetics method		
		log intercept method			log rate of formation method			Fixed time method			log rate of degradation method		
	Concentration levels →	Lower	Medium	Higher	Lower	Medium	Higher	Lower	Medium	Higher	Lower	Medium	Higher
Amount found¶ ($\mu g\ 10\ mL^{-1}$) ±SD	Series 1	10.17 ±0.08	40.23 ±0.24	69.66 ±0.82	10.20 ±0.09	30.06 ±0.23	50.51 ±0.33	10.21 ±0.11	40.40 ±0.19	69.96 ±0.44	40.44 ±0.35	79.90 ±0.38	120.70 ±0.93
	Series 2	10.24 ±0.05	40.54 ±0.31	70.37 ±0.56	10.24 ±0.07	30.30 ±0.36	49.87 ±0.37	10.28± 0.062	40.48 ±0.27	70.47 ±0.46	40.16 ±0.59	80.35 ±0.50	119.60 ±0.81
Relative SD (%)	Series 1	0.79	0.59	1.17	0.91	0.76	0.66	1.06	0.48	0.64	0.87	0.47	0.93
	Series 2	0.51	0.75	0.79	0.64	1.19	0.74	0.60	0.68	0.65	1.47	0.62	0.68
Intraserial variance		4.65×10^{-3}	7.47×10^{-2}	4.87×10^{-1}	6.49×10^{-3}	9.17×10^{-2}	1.24×10^{-1}	7.82×10^{-3}	5.62×10^{-2}	2.08×10^{-1}	2.36×10^{-1}	1.95×10^{-1}	7.60×10^{-1}
Interserial variance		1.71×10^{-3}	4.21×10^{-2}	2.02×10^{-1}	1.091×10^{-3}	2.09×10^{-2}	1.94×10^{-1}	1.66×10^{-3}	1.93×10^{-2}	1.10×10^{-1}	1.73×10^{-2}	8.13×10^{-2}	5.15×10^{-1}
Repeatability (%)		0.67	0.68	1.00	0.79	1.00	0.70	0.86	0.59	0.64	1.20	0.55	0.73
Overall mean		10.20	40.38	70.01	10.21	30.18	50.19	10.24	40.37	70.21	40.30	80.12	120.70
Overall variance		6.36×10^{-3}	11.69×10^{-2}	6.89×10^{-1}	7.56×10^{-3}	11.25×10^{-2}	3.17×10^{-1}	9.44×10^{-3}	7.54×10^{-2}	3.13×10^{-1}	2.53×10^{-1}	2.76×10^{-1}	1.28
Intermediate precision (%)		0.78	0.85	1.19	0.85	1.11	1.12	0.95	0.68	0.80	1.28	0.66	0.94

¶ For 10 independent analyses.

variance ratio test. The standard deviation and relative standard deviation were found to be satisfactory.

The accuracy of the proposed procedures was evaluated by the standard addition method as well as by the comparative studies of the proposed procedures with a reference method [23]. The recoveries, standard deviations and percent relative standard deviations are quite satisfactory wherease *t*–and *F*–values were found to be less than their theoretical values at 95% confidence level (Table 5.5 and 5.6).

In conclusion, the proposed procedures are economical, fast and suitable for the determination of diltiazem hydrochloride in pure form and pharmaceutical formulations.

Table 5.5. Standard addition method for the determination of diltiazem hydrochloride in dosage forms.

Formulation name	log Intercept method						Fixed time method					
	Amount (μg 10 mL^{-1})			Recovery (%) ±RSD(%)[a]	*SAE*[b]	Confidence limit[c]	Amount (μg 10 mL^{-1})			Recovery (%) ±RSD(%)[a]	*SAE*[b]	Confidence limit[c]
	taken	added	found ±SD[a]				taken	added	found ±SD[a]			
Angizem	30	30	60.15 ±0.33	100.25 ±0.55	0.135	0.271	30	30	60.00 ±0.45	100.00 ±0.75	0.184	0.370
Dilcardia	30	30	60.46 ±0.55	100.77 ±0.91	0.225	0.452	30	30	59.70 ±0.58	99.50 ±0.97	0.237	0.477
Diltime	30	30	59.65 ±0.62	99.42 ±1.04	0.253	0.510	30	30	60.15 ±0.50	100.25 ±0.83	0.204	0.411
	log rate of formation method						log rate of degradation method					
Angizem	20	20	40.37 ±0.43	100.93 ±1.07	0.176	0.354	50	50	99.99 ±0.30	99.99 ±0.30	0.122	0.247
Dilcardia	20	20	39.97 ±0.45	99.93 ±1.13	0.184	0.370	50	50	100.66 ±0.63	100.66 ±0.63	0.257	0.518
Diltime	20	20	40.27 ±0.52	100.68 ±1.29	0.212	0.428	50	50	100.83 ±0.75	100.83 ±0.74	0.306	0.617

[a] Mean ±S.D. for six determinations.
[b] *SAE*, standard analytical error.
[c] Confidence limit at 95% confidence level and five degrees of freedom ($t = 2.132$) [40].

Table 5.6. Comparison of the proposed methods with the reference method [30].

Pharmaceutical preparations	Labelled amount (mg)	log intercept method				Fixed time method				Reference Method	
		Recovery (%)[a]	RSD (%)[a]	*t*–value[b]	*F*–value[b]	Recovery (%)[a]	RSD (%)[a]	*t*–value[b]	*F*–value[b]	Recovery (%)[a]	RSD (%)[a]
Angizem	30	100.91	0.78	1.1481	1.07	101.19	0.66	1.7761	1.30	100.27	0.77
Dilcardia	30	99.11	0.94	1.5584	1.70	100.63	0.55	0.9519	4.90	100.09	1.21
Dilzem	30	100.01	1.00	0.6174	1.69	100.20	0.85	1.2848	1.24	99.58	0.76
		log rate of formation method				log rate of degradation method					
Angizem	30	100.59	0.69	1.5775	2.21	100.19	1.57	0.6284	2.35	99.69	1.03
Dilcardia	30	99.94	1.51	0.3243	2.64	99.73	1.70	0.7495	3.35	100.12	0.93
Dilzem	30	101.48	1.32	1.7178	1.32	99.82	1.35	0.7227	1.33	100.38	1.16

[a] Average of six independent analyses.

[b] Theoretical *t*– and *F*– values at 95% confidence level are 1.812 and 5.05, respectively [40].

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Spectrophotometric method for the determination of nifedipine with 4-(methylamino)phenol and potassium dichromate

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Abstract

A new simple, sensitive and reproducible spectrophotometric method for the determination of nifedipine in pure and dosage forms has been proposed It is based on the reduction of nifedipine with Zn/NH_4Cl, followed by coupling with *N*-methyl-1,4-benzoquinoneimine—the oxidation product of 4-(methylamino)phenol, to give a chromophore which absorbed maximally at 525 nm The experimental conditions were optimised and Beer's law was obeyed over the concentration range of 5–175 μg ml $^{-1}$ The molar absorptivity, detection limit, recovery and RSD were found to be 1.9×10^3 l mol $^{-1}$ cm $^{-1}$, 1 1 μg ml $^{-1}$, 99 7 100 5% and 0 3–0 8%, respectively The proposed method was compared favourably with the official B P method © 2002 Editions scientifiques et medicales Elsevier SAS All rights reserved

Keywords Nifedipine 4-(Methylamino)phenol, *N* Methyl 1,4 benzoquinoneimine, Potassium dichromate, Visible spectrophotometry

1. Introduction

Nifedipine, chemically dimethyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl) pyridine-3,5-dicarboxylate and pharmacologically a selective L-type slow calcium channel antagonist [1,2], is official in United States Pharmacopeia XXIII and British Pharmacopoeia [3,4] It is commonly used as an antihypertensive and potent arterial vasodilator in the management of angina and various other cardiovascular disorders [5] It is also used as a probe drug to assess cytochrome P-450 III A4 enzyme activity in vivo [6] Nifedipine decreases cyclic guanosine monophosphate in hypoxic lungs like inhaled nitric oxide, exhibits dose dependent depressive effect and causes some common side effects due to excessive vasodilation [7–9]

Several HPLC [6,10–12], reversed phase HPLC [13,14], HPTLC [15], GC [12,16–19] and voltammetric [20] methods have been reported for the assay of nifedipine and its related compounds in pharmaceuticals A variety of HPLC and GC methods are now widely used for the determination of nifedipine concentration in biological fluids because of their sensitivity and specificity These methods have adequate sensitivity to assay lower concentrations of the drug and hence use of these methods is justified when the sample matrix is complex and nifedipine concentration is low as in the case with the biological samples However, the sample matrix is usually less complex and analyte concentration levels are high in case of pharmaceutical analysis, hence it is required to develop a fast, simple and inexpensive method that can be adopted for routine analysis Therefore, spectrophotometry is still considered as a convenient and low cost technique

Nifedipine was assayed [21] in pharmaceutical formulations based on the reaction with 4-dimethylaminobenzaldehyde and subsequent determination at 310 nm Beer–Lambert's law was obeyed over the concentration range 5–60 μg ml $^{-1}$ Another spectrophotometric method has also been recommended for its determination involving the formation of blue complex with Folin–Ciocalteau reagent [22] A kinetic spectrophotometric method was described for its determination in

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dosage forms The method was based on its oxidation by potassium permanganate at neutral pH [23] In commercial dosage forms, UV-spectrophotometry has also been utilised for its estimation [24,25] The quantification of nifedipine in combined dosage forms were made using first derivative [12] and second derivative [27] spectra of their solutions in methanol and 0 1 N HCl, respectively

This work describes a new method for the determination of nifedipine in commercial dosage forms The method depends on the reduction of nitro group to hydroxylamino group which then reacted with *N*-methyl-1,4-benzoquinoneimine to form coloured product The reduction of nitro group of nifedipine to hydroxylamino derivative was studied with respect to heating time and concentration of Zn/NH_4Cl The effects of reagent s concentration, buffer and time on the formation of chromophore were investigated

2. Experimental

2 1 Apparatus

Spectral runs and absorbances were recorded on a Spectronic $20D^+$ spectrophotometer (Milton Roy, USA) pH-meter model L1-10T (Elico, India) was used to measure pH

2 2 Materials and reagents

(i) 0 1% ethanolic solution of reduced nifedipine was prepared by heating a mixture of 100 mg pure nifedipine (J B Chemicals and Pharmaceuticals Ltd , India) dissolved in 20 ml ethanol, 15 ml of 10% aqueous solution of ammonium chloride (Loba Chemie, India) and 2 g of zinc dust for 8 min on a water bath at 100 ± 1 °C The content was cooled at room temperature and diluted by adding 30 ml of ethanol It was filtered on Whatman no 42 filter paper and washed with ethanol The filtrate and washings were diluted to volume in a 100 ml volumetric flask The whole experiment was performed in dark and in amber-coloured glasswares This solution was stable at room temperature for about 4 days

(ii) A buffer solution was prepared by mixing appropriate volumes of 1 M hydrochloric acid (E Merck, India) and 1 M sodium acetate trihydrate (Ranbaxy Chemicals, India)

(iii) 0 2% aqueous solution of 4-(methylamino)phenol (Loba Chemie) was prepared in doubly distilled water It was always freshly prepared after every 5 h

(iv) 0 01 M potassium dichromate (Loba Chemie) was prepared by dissolving 0 7355 g in doubly distilled water and made up to 250 ml

2 3 Recommended procedure

Aliquots of 0 05–1 75 ml of reduced nifedipine (1 mg ml^{-1}) were transferred into a series of 10 ml standard volumetric flasks and then 2 5 ml of buffer solution (pH 2 9), 1 45 ml of 0 2% 4-(methylamino)phenol and 1 2 ml of 0 01 M potassium dichromate were added to each flask successively The solutions were allowed to stand at room temperature for 18 min and then made up to the mark with doubly distilled water The absorbance values of the final coloured solutions were measured at 525 nm against a reagent blank The amount of the drug was computed from a Beer–Lambert's plot

2 4 Analysis of pharmaceutical formulations

Ten tablets of nifedipine (each claiming 10 mg) were finely powdered and thoroughly mixed The powdered mixture was transferred in a conical flask 20 ml of ethanol was added and gently shaken for 2–3 min Then 15 ml of 10% ammonium chloride solution and 2 g of zinc dust were added and heated on a water bath for 8 min After cooling at room temperature, the mixture was added with 30 ml of ethanol and filtered on Whatman filter paper no 42 in a 100 ml standard volumetric flask The residue was washed with enough ethanol and finally made up to the mark Nifedipine content was determined using the recommended procedure

3. Investigation of stability

The stability of nifedipine under the experimental conditions was investigated by incubating 120 μg ml^{-1} in distilled water or ethanol containing common excipients at 30 °C for 3 h in the absence of all lights At regular time interval the concentration of nifedipine was determined by the proposed and reference methods [23]

4. Results and discussion

The nitro compounds undergo reduction by catalytic hydrogenation in the presence of metals (Zn, Fe, Sn) and other suitable reagents like, HCl, NH_4Cl, NaOH or KOH Under the proposed condition, nifedipine is reduced to hydroxylamino derivative by Zn/NH_4Cl

The primary aromatic amines react with 4-(methylamino)phenol and an oxidising agent such as dichromate [28], *N*-bromosuccinimide [29], peroxydisulfate [30] or iodylbenzoate [31] to form a purple red product It is believed that on oxidation 4-(methylamino)phenol produces *N*-methyl-1,4-benzoquinoneimine The primary aromatic amines react with *N*-methyl-1,4-benzo-

quinoneimine to form chromophore which absorbed maximally at 520–530 nm In this study the reaction of hydroxylamino derivative of nifedipine with 4-(methylamino)phenol and potassium dichromate may be assumed to proceed in an analogous manner as the product absorbed maximally at 525 nm [28] The stoichiometric ratio of hydroxylamino derivative to *N*-methyl-1,4-benzoquinoneimine was determined by Job's method [32] and was found to be 2 1 (Fig 1) The chromophore formed was found to be positively charged at pH 2 9 as it was adsorbed on cation-exchange resin beads The possible reaction sequence is presented in Scheme 1

The optimum conditions for reduction of nifedipine to hydroxylamino derivative were established via a number of preliminary experiments The effect of variables on the reduction of the drug was studied by taking a separate 10 ml aliquot of 0 1% nifedipine solution

4 1 Effect of heating time

The aliquot of nifedipine was mixed with 200 mg of zinc dust and 1 5 ml of 10% ammonium chloride solution and the content was heated on a water bath at 100 ± 1 °C One millilitre of aliquot of this solution was subjected to colour development The results showed that the intensity of the colour reached its maximum at 7 min of heating and remained unchanged even after 10 min Therefore, a heating time of 8 min was recommended for reduction

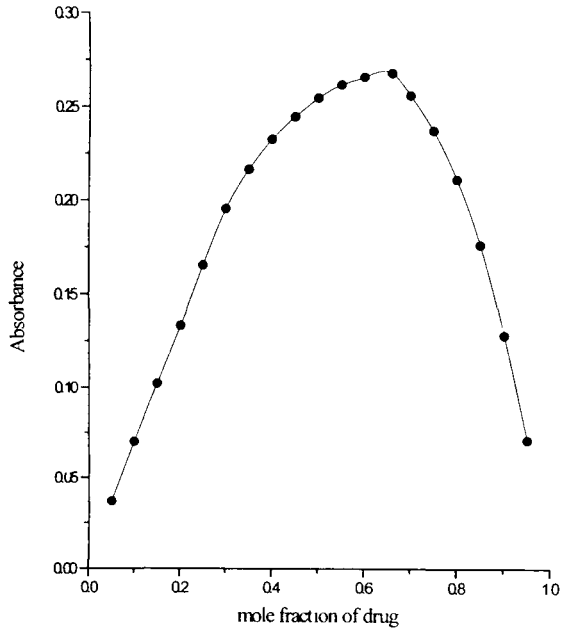


Fig 1 Job's plot for hydroxylamino derivative of nifedipine and N methyl 1 4-benzoquinoneimine (each 2 89 × 10^{-3} M)

4 2 Effect of zinc dust

The effect of the amount of zinc dust on the reduction of 10 mg nifedipine in the presence of 1 5 ml of 10% ammonium chloride solution was studied It was observed that the absorbance of the coloured solution increased up to 150 mg of zinc dust and then remained constant at higher amounts Hence, 200 mg of zinc dust was taken as optimum value for reduction

4 3 Effect of ammonium chloride solution

To study the effect of the concentration of ammonium chloride solution on the reduction of nifedipine 10 ml of 0 1% nifedipine was mixed with 200 mg of zinc dust and varying volumes of 10% ammonium chloride solution The content was heated on a water bath at 100 ± 1 °C for 8 min A plot of absorbance versus volume of ammonium chloride solution showed that the highest absorbance was obtained with 1 25 ml and remained constant beyond this volume Therefore, 1 5 ml of 10% ammonium chloride solution was taken to reduce the drug for further studies

The optimum conditions for the development of the proposed method were established by varying the parameters one at a time and observing the effects produced

4 4 Effect of buffer solution

A constant absorbance was observed in the pH range 2 75 3 1 In this study, therefore, 2 5 ml of pH 2 9 buffer solution was used throughout the experimental investigations

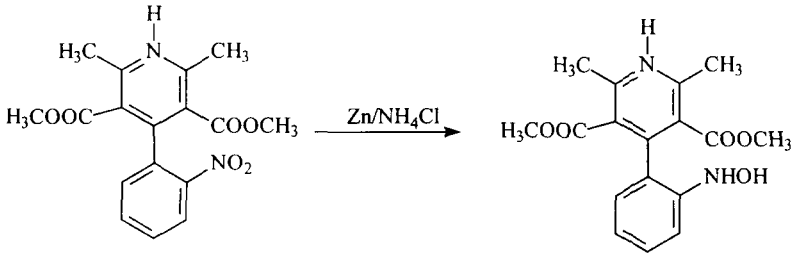
4 5 Effect of time

To 1 ml of 0 1% reduced drug 2 5 ml of buffer solution (pH 2 9), 1 45 ml of 0 2% 4-(methylamino)phenol solution and 1 2 ml of 0 01 M potassium dichromate were added and kept at room temperature (25 °C) for colour development The intensity of the colour was reached to maximum after 15 min and remained constant for 1 h The coloured product was diluted to 10 ml with distilled water and absorbance was measured at 525 nm against a reagent blank after 18 min

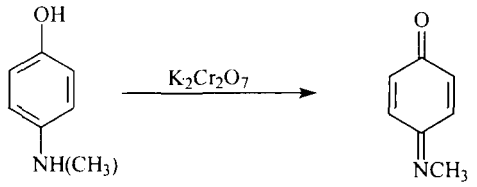
4 6 Effect of 4-(methylamino)phenol solution

1 ml of 0 1% reduced drug, was mixed with 2 5 ml of buffer solution (pH 2 9), varying volumes of 0 2% 4-(methylamino)phenol and 1 2 ml of 0 01 M potassium dichromate and the contents were allowed to stand at room temperature for 18 min The results showed that a constant absorbance was found in the range of 1 4–

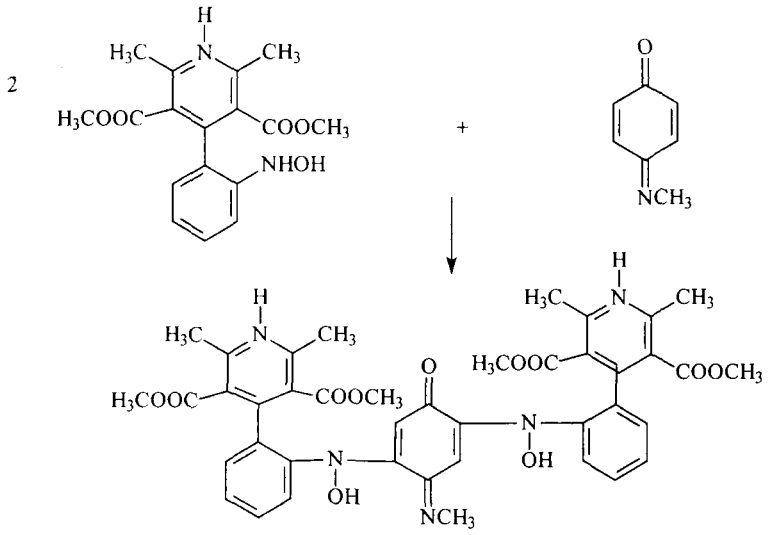
(a) Reduction of nifedipine to hydroxylamino derivative



(b) Formation of 4-N-methylbenzoquinoneimine:



(c) Coupling with hydroxylamino derivative of drug:-



Scheme 1.

1.5 ml. Therefore 1.45 ml of 0.2% 4-(methylamino)phenol was used in all the subsequent works.

4.7. Effect of potassium dichromate solution

In order to study the effect of potassium dichromate concentration, the reaction was carried out in a series of 10 ml volumetric flasks containing 100 μg ml^{-1} reduced drug, 2.5 ml of buffer solution (pH 2.9), 1.45 ml of 0.2% 4-(methylamino)phenol solution. This was followed by different volumes of 0.01 M potassium dichromate ranging from 0.3 to 1.5 ml. The results indicate that the highest intensity and reproducible results are obtained on using 1 ml of 0.01 M potassium dichromate. Therefore, 1.2 ml of this reagent was used throughout this work.

4.8. Analytical data

Under the optimum experimental conditions, a calibration curve was constructed by plotting absorbance at 525 nm versus concentration. Beer's law was obeyed within a concentration range of 5–175 μg ml^{-1}. Regression analysis using the method of least squares was

made to evaluate the slope, intercept and correlation coefficient The linear regression equation and correlation coefficient are $A = 1\,3 \times 10^{-3} + 5\,3 \times 10^{-3} C$ (A, absorbance at 525 nm, C, concentration in μg ml^{-1}) and $r = 0\,9999$ which indicates an excellent linearity The molar absorptivity, detection limit and variance [33], standard deviations of intercept and slope [34] were found to be $1\,9 \times 10^{3}$ l mol^{-1} cm^{-1}, 1 1 μg ml^{-1} and $1\,2 \times 10^{-5}$, $1\,9 \times 10^{-3}$ and $1\,6 \times 10^{-5}$, respectively The small value of variance marks the negligible scattering of the experimental data points from the line of regression

The error, S_c, in the determination of a given concentration of nifedipine was calculated by statistical analysis of calibration data using the relation [35]

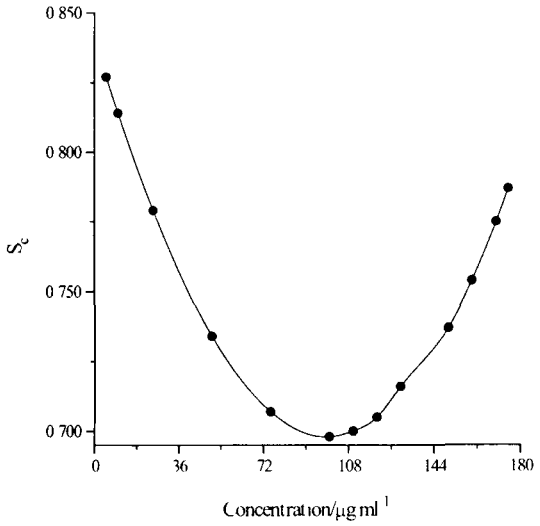


Fig 2 Errors (S_c) in the determination of the concentration of nifedipine

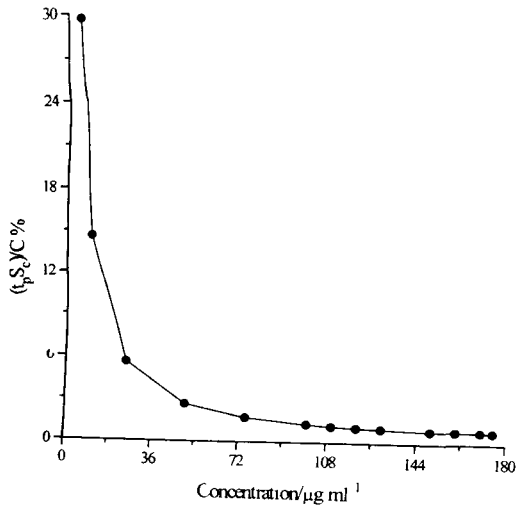


Fig 3 Variation of confidence limit at 95% confidence level in the form of percent uncertainty on the concentration

$$S_c = \frac{S_o}{b}\left(1 + \frac{1}{n} + \frac{(A - A')^2}{b^2(\Sigma C^2 - nC'^2)}\right)^{1/2}$$

where C' and A' are average concentration and average absorbance values, respectively, for n standard solutions Fig 2 shows the graph of S_c against the concentration of nifedipine The value of S_c reached a minimum when the actual absorbance was equal to the average absorbance in the calibration graph Thus the minimum error was found in the determination of about 100 μg ml^{-1} nifedipine The value of S_c also allows us to establish the confidence limits at a selected level of significance [35] The results are shown in Fig 3 in the form of percent uncertainty on the concentration at 95% confidence level It is apparent from the figure that the relative uncertainty on the concentration can be calculated directly over the full range of the concentration tested and hence, confidence limit can be established

The stability experiment conducted in the presence of commonly encountered excipients such as starch, talc lactose and magnesium stearate revealed the fact that under the conditions no degradation of nifedipine was detected The drug and its photodegradation product 4-(2-nitrosophenyl)-pyridine homologue may undergo reduction with Zn/NH_4Cl to yield hydroxylamino derivative However, the determination was done under a condition where contact with light was completely avoided

The reproducibility of the method was checked by ten replicate determinations at the concentration levels of 60, 100, 120 and 150 μg ml^{-1} The percent relative standard deviations were found to vary between 0 3 and 0 8

The accuracy of the method was demonstrated by recovery experiments which were carried out by adding a fixed amount of pure drug to the preanalysed formulations The analytical results obtained from these investigations are summarised in Table 1 which indicates that common additives and excipients did not interfere with the determination The percent relative standard deviations can be considered to be very satisfactory

The proposed method for assay of nifedipine in dosage forms was compared favourably with other existing UV–visible spectrophotometric methods (Table 2) It is evident from the table that the method has advantages of wider linear dynamic range and high precision (%RSD = 0 3–0 8 and 0 4–0 7 for pure and dosage forms, respectively)

Some commercial dosage forms were successfully analysed by the proposed method and official B P method [4] The results (Table 3) were compared statistically by Student's t-test and variance ratio F-test which indicates that there is no significant difference between the methods compared

Table 1
Spectrophotometric determination of nifedipine in pharmaceutical formulations by standard addition method

Preparations	Amount taken (μg ml $^{-1}$)	Amount added (μg ml^{-1})	Amount found (μg ml^{-1})	Recovery (%)	RSD (%)
Adalat Retard-10	30	30	60 1	100 1	0 8
	40	60	99 5	99 5	0 6
	80	40	120 4	100 3	0 5
Calcigard-10	30	30	59 5	99 2	1 0
	40	60	99 8	99 8	0 8
	80	40	120 7	100 6	0 5
Nicardia Retard-10	30	30	59 5	99 2	0 7
	40	60	100 4	100 4	0 4
	80	40	120 5	100 4	0 5

Table 2
Comparison of the proposed method with existing spectrophotometric methods for assay of nifedipine in pharmaceutical formulations

Reagents	λ_{max} (nm)	Linear dynamic range (μg ml $^{-1}$)	Recovery (%)	RSD (%)	References
Potassium permanganate	530	18–44	99 5 101 3	1 5	[23]
3,4,5-Trimethoxybenzaldehyde	365	10–70	100 2 102 9	1 5	[26]
4-Dimethylaminobezaldehyde	310	5–60	97 8 98 5		[21]
Extractive U V [a]	237	0–10	97 8 98 9		[25]
Ethanol and phosphate buffer saline	340		99 7–99 9		[24]
Derivative U V	400	4–12	98 5–101 3	1 4	[12]
4-*N*-Methylaminophenol and dichromate	525	5–175	99 7–100 5	0 6	This work

[a] Extracted into chloroform and the solvent was evaporated to dryness Finally, the residue was dissolved in distilled water

Table 3
Spectrophotometric determination of nifedipine in pharmaceutical formulations by the proposed method and B P method

Preparations	Nominal composition (mg)	Proposed method			Reference method			F-value[c]
		Recovery[a] (%)	RSD[a] (%)	t[b]	Recovery[a] (%)	RSD[a] (%)	t[b]	
Adalat Retard-10	10	99 9	0 6	0 3923	100 1	0 4	1 3975	2 0306
Calcigard 10	10	100 2	0 7	0 6675	100 2	0 5	0 8856	2 2168
Nicardia Retard-10	10	99 9	0 4	0 520	100 4	0 5	1 9876	1 0952

[a] Five independent analyses
[b] t, the value at 95% confidence level is 2 132 [36]
[c] F, value at 95% confidence level is 6 39 [36]

Acknowledgements

The authors are grateful to Professor M Ilyas, Chairman, Department of Chemistry, Aligarh Muslim University, Aligarh, for providing research facilities. The authors wish to express their gratitude to M/s J.B. Chemicals & Pharmaceuticals Ltd, India for sample of pure nefidipine.

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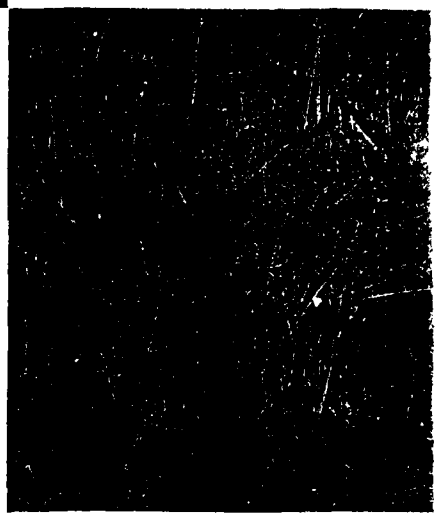

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ORIGINAL PAPER

Nafisur Rahman Md Nasrul Hoda

Spectrophotometric determination of verapamil hydrochloride in drug formulations with chloramine-T as oxidant

Received 15 January 2002 / Revised 17 July 2002 / Accepted 17 July 2002 / Published online 7 September 2002

Abstract A new spectrophotometric method is described for the determination of verapamil hydrochloride, based on its oxidation with chloramine-T in hydrochloric acid medium It produces a yellow colored compound with maximum absorbance at 425 nm Beer's law was obeyed in the concentration range 0–340 μg mL^{-1} with molar absorptivity 2×10^3 L mol^{-1} cm^{-1} and RSD 0 3–0 82% All variables were studied to optimize the reaction conditions No interferences were observed from the common excipients present in the formulations The method has been applied successfully to the determination of the drug in pharmaceutical preparations Statistical comparison of the results with those from the reference method reveals excellent agreement and confirms that accuracy and precision are not significantly different

Keywords Verapamil hydrochloride Chloramine-T Spectrophotometry Pharmaceutical formulations

Introduction

Verapamil hydrochloride, belonging to phenylalkylamine group of calcium channel blockers, is chemically 5-[*N*-(3,4-dimethoxyphenyl ethyl)methylamino]-2-(3,4 dimethoxyphenyl)-2-isopropyl valeronitrile hydrochloride [1] The drug is officially listed in British, United States, and Indian Pharmacopeias [2, 3, 4] It is a potent antihypertensive agent with significant depressant effects and has been recommended for intravenous therapy of supraventricular tachyarythmias [5]

The drug has been determined in biological fluids and in pharmaceutical dosage forms by a variety of analytical techniques such as high-performance liquid chromatography [6, 7, 8, 9, 10, 11], gas–liquid chromatography [12], capillary gas chromatography [13], high-performance thin layer chromatography [14], potentiometry [15] and potentiometry–conductometry [16] The visible spectrophotometric methods are the instrumental methods of choice and have practical and significant economic advantages over the other methods In the literature few spectrophotometric methods have been reported, these are usually based either on extractable ion-pair complex formation with bromophenol blue, bromocresol purple, bromocresol green, bromothymol blue and methyl orange [17], solochrome black-T, solochrome dark blue, solochrome cyanine R and fast sulfone FF [18], erioglaucine and indigocarmine [19], alizarin red-S [20] or on charge-transfer complex formation with polyhalo- or polycyanoquinones [21, 22] and azo dyes [23] Some of these methods, however, suffer from disadvantages such as low sensitivity, lack of selectivity, and complexity

Chloramine-T is a strong oxidant in both acidic and alkaline media (E_{red}=1 138 at pH 0 65 and 0 5 at pH 12) [24, 25] In acidic aqueous solution, chloramine-T is thought to exist in a complex series of equilibria [26] which indicates that the probable oxidizing species in acidified chloramine-T is hypochlorous acid

Chloramine-T was initially introduced as a disinfectant and antiseptic but is now widely used as an oxidant for a variety of organic functional groups [27] It has been used as a reagent for the spectrophotometric determination of *p*-aminobenzenesulfonamides [28] and sulfamethoxazole, tetracycline hydrochloride, amidopyrine, nifurtimox, and isoniazid [29] which involves addition of excess chloramine-T and determination of unreacted reagent Chloramine-T is also used in titrimetric determination, the end point being detected with either a visual indicator [28, 30, 31, 32] or potentiometrically [31]

This work describes the spectrophotometric determination of verapamil hydrochloride on the basis of its oxidation with chloramine-T in acidic medium to produce a yellow chromophore

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Experimental

Apparatus

A Spectronic 20D+ spectrophotometer (Milton Roy, USA) with matched glass cuvettes was used for spectral runs and absorbance measurements

Reagents

Verapamil hydrochloride (Sigma USA) solution (0 4%) was prepared in methanol (AR grade, S D Fine Chemicals, India) and was further diluted according to the need Solution of chloramine-T (E Merck, India 1%) and hydrochloric acid (E Merck, 5 mol L^{-1}) were prepared in doubly distilled water

Recommended procedure

Different concentrations of verapamil hydrochloride, up to 340 μg mL^{-1}, were pipetted (1 mL) into 10 mL volumetric flasks Chloramine T solution (1% 2 5 mL) and HCl (5 mol L^{-1}, 6 mL) were added to each flask and the solutions were diluted to volume with doubly distilled water The contents were mixed well and kept at room temperature for 15 min Absorbance was measured at 425 nm against the reagent blank prepared simultaneously but omitting the drug The amount of drug in each sample was calculated either from the calibration graph or the regression equation

Analysis of formulations

Ten tablets of verapamil hydrochloride, equivalent to 400 mg of pure drug, were ground to fine powder The whole mass was stirred in methanol and filtered through Whatman no 42 filter paper into a 100-mL volumetric flask The residue was washed well with methanol The filtrate and washings were diluted to 100 mL This solution was diluted according to the need and analyzed by the recommended procedure

Results and discussion

Chloramine-T is well known oxidant, its oxidative behavior resembles to that of hypohalites Bishop and Jennings [33], Morris et al [34], and Higuchi and Hussain [35] have studied the equilibria involved in acidified chloramine-T solution and suggested the formation of hypochlorous acid This reacts with verapamil hydrochloride to form the relevant oxidation products It is also known that *N*-bromosuccinimide reacts with tertiary amines [36] In such studies, a methyl or methylene group attached to nitrogen was required and >N–CH_2 linkage was cleaved preferentially giving yellow colored products In a similar fashion it is believed that verapamil is oxidized by hypochlorous acid, in which the >N–CH_2 bond is cleaved, resulting in the formation of aldehyde and secondary amine. The oxidation product absorbs maximally at 425 nm (Fig 1) Therefore, based on the literature background and our findings, the reaction mechanism was proposed and given in Scheme 1

The optimum conditions for the assay of verapamil hydrochloride were established via a number of preliminary experiments

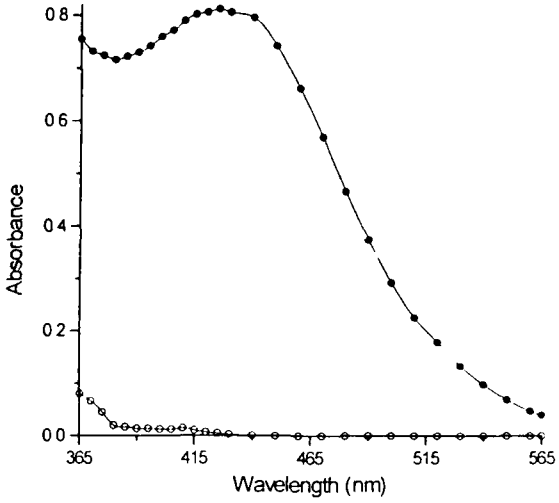
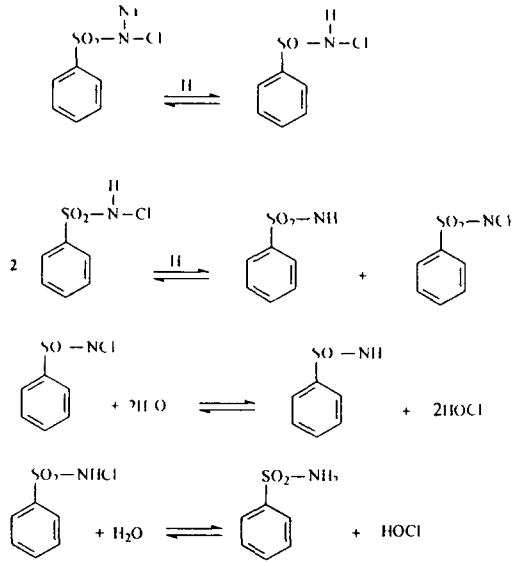
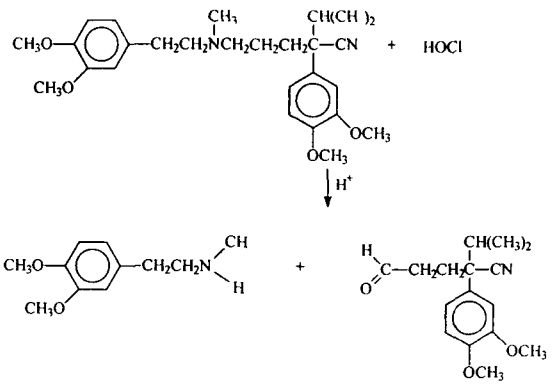


Fig. 1 Absorption spectra of the oxidation product of verapamil hydrochloride (*filled circles*) and reagent blank (*empty circles*)

(1) Formation of hypochlorous acid



(2) Oxidation of verapamil by hypochlorous acid



Scheme 1 Formation of hypochlorous acid and oxidation of verapamil

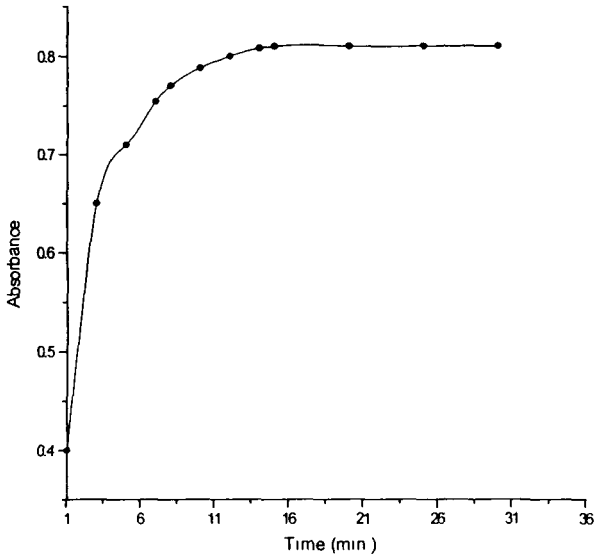


Fig. 2 Effect of time on the oxidation of verapamil hydrochloride

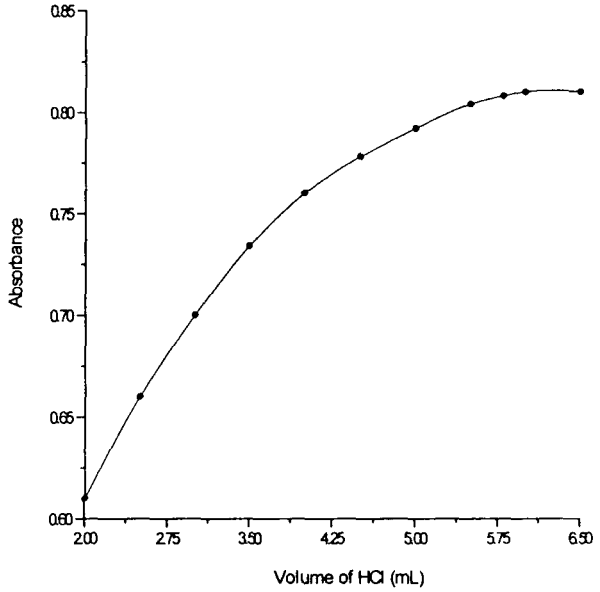


Fig. 4 Effect of the volume of 5 mol L^{-1} HCl on the oxidation of verapamil hydrochloride

Effect of time

To investigate the effect of time on color development, 1 mL of 0.2% verapamil hydrochloride was pipetted into a 10 mL volumetric flask, 2.5 mL of 1% chloramine-T and 6 mL of 5 mol L^{-1} HCl were added, and the mixture was diluted to volume with doubly distilled water. The absorbance was recorded as a function of time. The results showed (Fig. 2) that absorbance became constant after 13 min and remained unchanged up to 20 min. Thus absorbance was measured within the stability period.

Effect of chloramine-T concentration

To 1 mL of 0.2% verapamil hydrochloride, different volumes (0.5–3.0 mL) of 1% chloramine-T and 6 mL of 5 mol L^{-1} HCl were added. The colored product was diluted to 10 mL with doubly distilled water and absorbances were measured against the corresponding reagent blank after 15 min. The results (Fig. 3) showed that the highest absorbance was obtained with 2.25 mL and remained constant for larger amounts of chloramine-T. Thus 2.5 mL of 1% chloramine-T was added for color development.

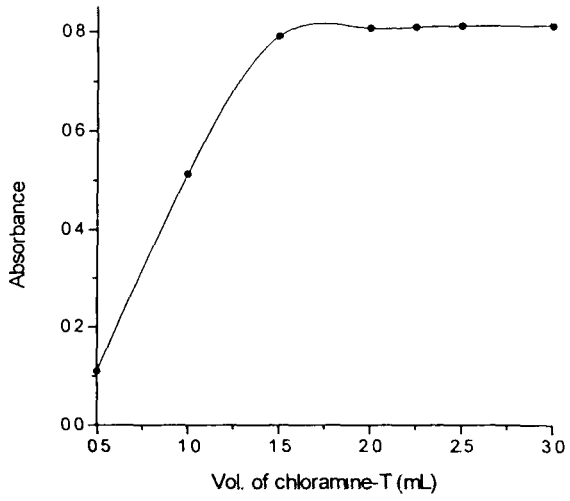


Fig. 3 Effect of volume of 1% chloramine-T on the oxidation of verapamil hydrochloride

Effect of hydrochloric acid concentration

To study the effect of hydrochloric acid concentration the reaction was performed in a series of 10-mL volumetric flasks containing 200 μg mL^{-1} verapamil hydrochloride, 2.5 mL of 1% chloramine-T, and different volumes of 5 mol L^{-1} HCl (2.0–6.5 mL). It is apparent from Fig. 4 that the maximum absorbance was obtained for 5.8 mL of 5 mol L^{-1} HCl, beyond which the absorbance became constant. Thus, 6 mL of 5 mol L^{-1} HCl was used throughout the experiment.

Analytical data

Under the optimum experimental conditions the main merits of the procedure for the determination of verapamil hydrochloride were established by least squares treatment of the results. The absorbance at 425 nm was found to be linearly dependent on the concentration of verapamil hydrochloride up to 340 μg mL^{-1} with a molar absorptivity of 2×10^3 L mol^{-1} cm^{-1}. Regression analysis of Beer's law plot was performed to evaluate intercept, slope, and correlation coefficient (r) and the values were found to be 0.29×10^{-3}, 4.05×10^{-3}, and 0.9999, respectively which yielded the regression equation, $A=0.29\times10^{-3}+4.05\times10^{-3}$ C (where A is the absorbance and C is the concentration of verapamil hydrochloride in μg mL^{-1}). The detection limit for

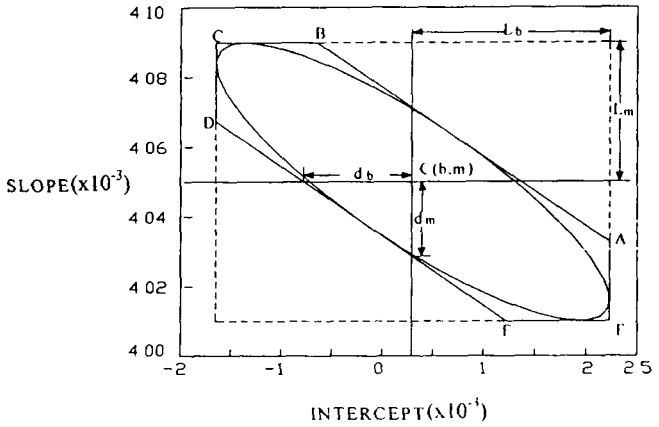


Fig. 5 Plot of joint confidence region (at p=0.05) for the slope and intercept of the line of regression for the determination of verapamil hydrochloride

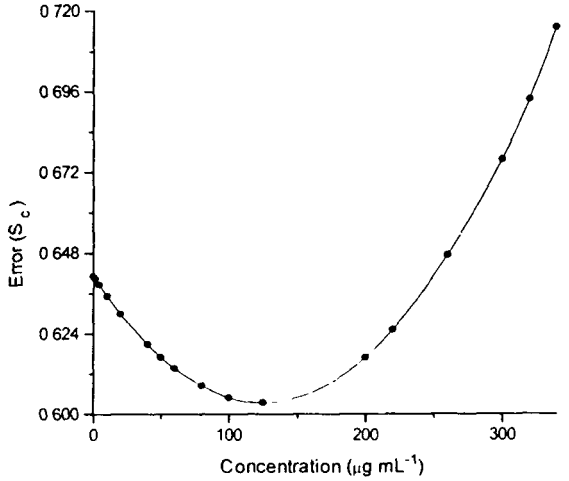


Fig. 6 Error (S_c) in the determination of the concentration of verapamil hydrochloride

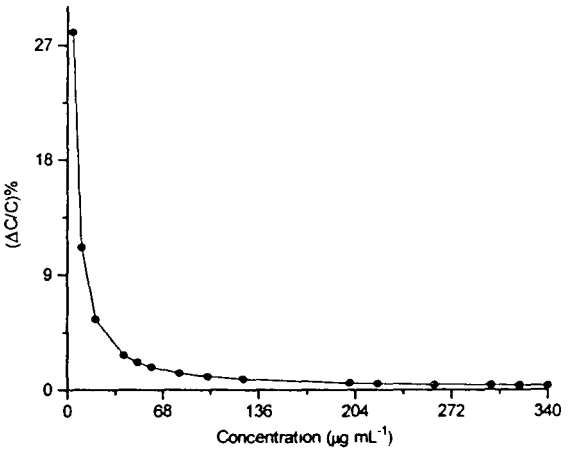


Fig. 7 Variation in the confidence limit, at 95% confidence level, for the determination of verapamil hydrochloride

the proposed method was 0.97 μg mL^{-1}, computed by use of the equation [37]:

$$\text{Detection limit} = \sqrt{S_o^2 \frac{n-2}{n-1}} \frac{t}{b} \quad (1)$$

where S_o^2=variance, n=number of samples, t=Student's t-value at 95% confidence level and b=slope of line of regression. The high value of correlation coefficient and small value of the intercept on the ordinate, which was close to zero, validated the linearity of calibration curve whereas detection limit and slope indicated the good sensitivity of the method. Also, the small amount of scattering of the experimental data points around the line of regression was confirmed by the small value of variance, i.e. 5.33×10^{-6}.

There was also strong correlation between slope and intercept; this was established by the 95% joint confidence region drawn for them [38]. It is evident from the Fig. 5 that the joint confidence region is bounded by an ellipse with the point of best fit as its center. It can also be seen that the points with an intercept of zero fell well within the ellipse and thus confirmed there was no significant deviation from zero.

Regression analysis of the calibration data also make it possible to evaluate the error, S_c, in the determination of a given concentration of verapamil hydrochloride [39]. Figure 6 shows the graph of S_c against the concentration of verapamil hydrochloride. The error is minimum when the actual absorbance is equal to the average absorbance value in the calibration graph, which corresponds to 125 μg mL^{-1}. This statistical treatment can be used to establish the confidence limit, at the selected level of confidence, for determination of unknown concentrations, by use of the equation [40].

$$C_i \pm \frac{t_p S_o}{b} \left(1 + \frac{1}{n} + \frac{(y - \bar{y})^2}{b^2 \left(\sum C^2 - n\bar{C}^2 \right)} \right)^{1/2} = C_i \pm \Delta C \quad (2)$$

where $\bar{y}$ and $\bar{C}$ are average absorbance and concentration values respectively for n standard specimens. The results are shown graphically in Fig. 7 in the form of the percentage uncertainty in the concentration at the 95% confidence level and used to calculate the relative uncertainty in the concentration over the full range of concentration tested. It is, therefore, a guide to establish the confidence limit.

To test the precision and accuracy of the proposed method ten successive determinations of 200 μg mL^{-1} verapamil hydrochloride were performed. The percentage relative standard deviation (%RSD) and error (%Er) were found to be 0.24 and 0.22 respectively. The results, therefore, indicated that the precision and accuracy of the method were satisfactory. The commonly encountered excipients in the pharmaceutical dosage forms did not interfere.

As an additional demonstration of accuracy, recovery experiments were performed by adding a fixed amount of verapamil hydrochloride to a preanalyzed tablet. The results are shown in Table 1. It is apparent from the table that results were reproducible with low relative standard deviations (0.3–0.82%) and mean recoveries were in the range of 99.4–100.3%.

Table 1 *Determination of* verapamil hydrochloride in dosage forms by the standard addition method

Pharmaceutical preparation	Amount taken (μg mL^{-1})	Amount added (μg mL^{-1})	Total amount found (μg mL^{-1})[a]	Recovery (%)[a]	RSD (%)[a]
Calaptin 40	25	25	49 85	99 71	0 73
	150	150	300 20	100 10	0 30
Isoptin 40	25	25	50 15	100 30	0 82
	150	150	300 94	100 30	0 45
Vasopten-40	25	25	49 10	99 40	0 75
	150	150	300 2	100 10	0 67

[a]Five independent analyses

Table 2 Analysis of pharma ceutical preparations by the pro posed and reference methods

Pharmaceutical preparation	Labeled amount (mg)	Proposed method		Reference method [22]		t_{calc}[b]	F_{calc}[b]
		Recovery (%)[a]	RSD (%)[a]	Recovery (%)[a]	RSD (%)[a]		
Calaptin	40	99 76	0 49	99 65	0 67	0 3154	1 9
Isoptin	40	100 12	0 41	99 88	0 49	0 8253	1 42
Vasopten	40	99 63	0 38	99 88	0 77	0 6720	4 16

[a]Average from five indepen dent analyses
[b]Theoretical t and F values at 95% confidence level are 1 86 and 6 39, respectively [41]

Table 3 Comparison of results from the proposed method with those from other spectrophoto metric methods for the deter mination of verapamil hydro chloride in pharmaceutical for mulations

Reagent	λ_{max} (nm)	Beer's law limit (μg mL^{-1})	RSD (%)	Reference
Erioglaucine[a]	627	1 3–5 3	1 45	[19]
Indigocarmine[a]	602	33–130	1 53	[19]
Bromocresol purple[a]	420	4–24		[17]
Chromotrope 2 B	530	5–59	-	[23]
Chromotrope 2 R	546	5–59	-	[23]
Solochrome dark blue[a]	528	10–38	1 53	[18]
Solochrome cyanine R[a]	445	8–30	1 24	[18]
Chloramine T	425	0–340	0 3–0 82	This work

[a]Extractive method

The method was successfully applied to the determination of verapamil hydrochloride in tablets available locally Satisfactory results (Table 2) were obtained for the recovery of drug and were in a good agreement with the label claim The results of the proposed method were statistically compared with those obtained by the reference method [22] The calculated Student's t- and F-values were less than the theoretical values for 95% confidence level Statistical evaluation indicated there was no significant difference between the methods compared (Table 3)

Conclusion

The proposed method is a direct oxidative method for the determination of verapamil hydrochloride in pure and dosage forms which does not require elaborate treatment of the analyte and tedious extraction of the chromophore produced The proposed method is advantageous when compared with other visible spectrophotometric methods because the linear range of estimation is wider and the percentage relative standard deviation is lower (0 30–0 82) This is a decisive advantage, because commercial dosage forms of verapamil hydrochloride contain larger amounts The method is sensitive enough to enable determination of as little as 0 97 μg mL^{-1} of the drug In conclusion, the method is simple, sensitive, precise, accurate, economical, and appropriate and can be used for the routine analysis of verapamil hydrochloride in quality-control laboratories

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Short communication

Validated spectrophotometric methods for the determination of amlodipine besylate in drug formulations using 2,3-dichloro 5,6-dicyano 1,4-benzoquinone and ascorbic acid

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Abstract

Two simple and sensitive spectrophotometric methods have been proposed for the determination of amlodipine besylate either in pure form or in pharmaceutical formulations. The first method is based on the charge transfer complexation reaction of the drug with 2,3-dichloro 5,6-dicyano 1,4-benzoquinone (DDQ) to give coloured product having maximum absorbance at 580 nm. The second procedure depends on the measurement of purple red colour produced by the interaction of drug with ascorbic acid in *N*,*N*-dimethylformamide medium (DMF) which absorbed maximally at 530 nm. Under the optimized experimental conditions, Beer's law was obeyed in the concentration ranges of 1–125 and 10–140 μg ml^{-1} with DDQ and ascorbic acid, respectively. Both the methods were applied successfully for the analysis of amlodipine besylate in dosage forms. Results of analyses were validated statistically and through recovery studies.

Keywords: Amlodipine besylate; 2,3-Dichloro 5,6-dicyano 1,4-benzoquinone; Ascorbic acid; Spectrophotometry

1. Introduction

Amlodipine besylate is listed in Martindale, The Extra Pharmacopoeia and European Pharmacopoeia [1,2] which is chemically (4*R*,*S*)-3-ethyl 5-methyl 2-(2-amino-ethoxy-methyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl pyridine-3,5-dicarboxylate monobenzene sulphonate, approved for the treatment of variant and stable angina and hypertension. It is relatively a new long acting calcium channel blocker with slow onset of vasodilatory action [3,4]. It may also be used for dilated cardiomyopathy and exhibits ameliorating effects on plasma and myocardial catecholamines with a significant reduction of calcium deposition [5,6]. In addition to calcium channel blocking ability, amlodipine also inhibits vascular smooth muscle cell growth through interactions with targets other than L-type calcium channels [7]. Amlodipine is more selective for arterial vascular smooth muscle than cardiac tissues. Due to these important pharmacological responses, develop-

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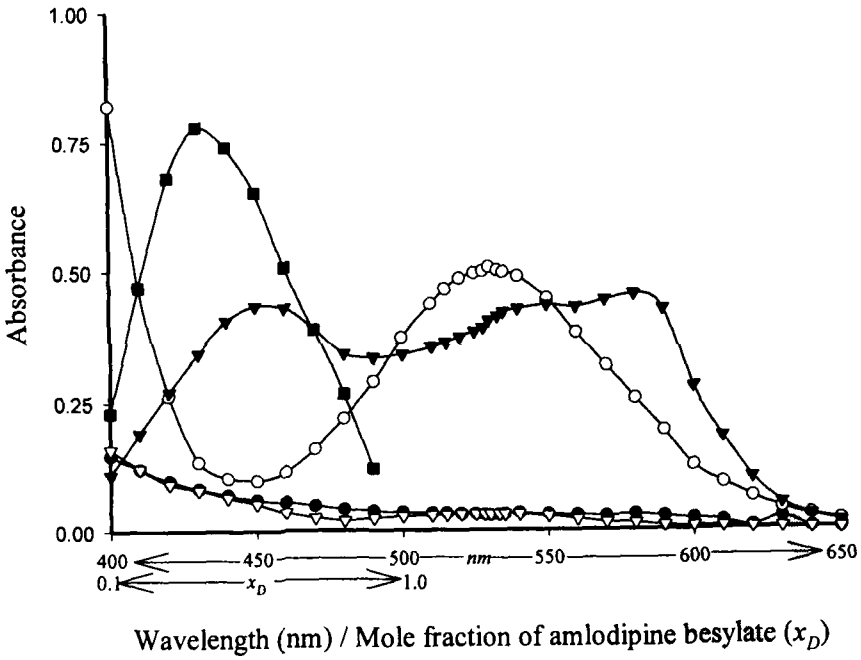


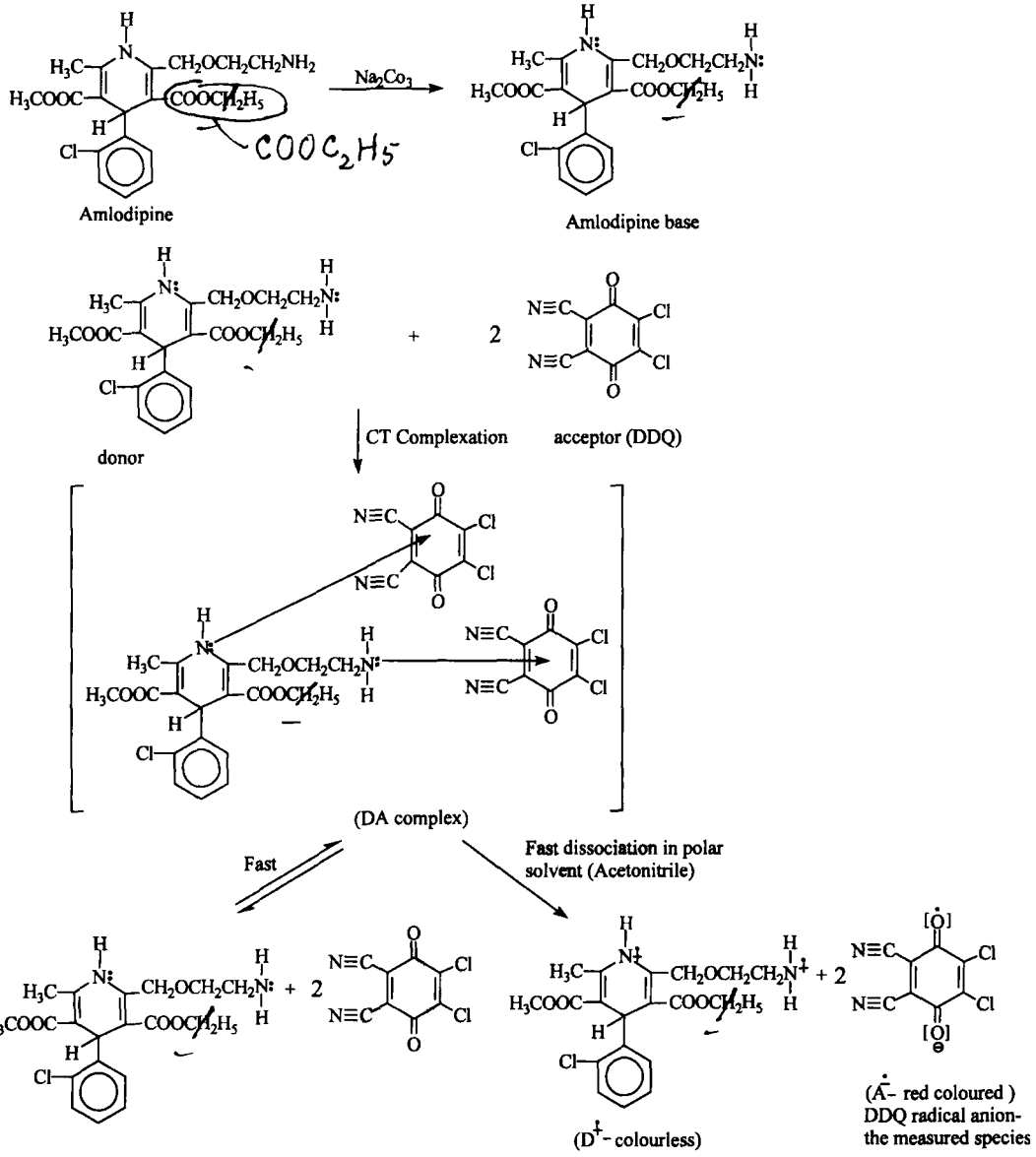
Fig. 1. Absorption spectra of the reaction products of amlodipine besylate with DDQ (▼) and ascorbic acid (○) and their respective reagent blanks. Job's plot for amlodipine–DDQ complex (■).

ment of sensitive and accurate methods for the determination of amlodipine besylate is desired.

Different methods for the quantification of amlodipine besylate have been reported which include high performance liquid chromatography [8–13], reversed phase high performance liquid chromatography [2,14–16], high performance thin layer chromatography [17 20], gas chromatography [21], gas chromatography–mass spectrometry [22], liquid chromatography with tandem mass spectrometry [23] and fluorimetry [24]. Though these methods are sensitive enough, they are expensive and not easily manageable. On the other hand, spectrophotometry is still the technique of choice since it is sensitive, economical, rapid and more easily manageable for third world countries.

Few spectrophotometric methods have been reported for the assay of amlodipine besylate based on extractable ion-pair complexes [25 29], oxidative coupling with 3-methyl 2-benzothiazolinone hydrazone hydrochloride [30], with sodium hydroxide [31], derivative spectroscopy [32,33], simultaneous multicomponent mode of analysis [34] and charge transfer complexation with *p*-chloranilic acid [35] and chloranil [36]. It has also been determined based on the reaction of $-NH_2$ group with ninhydrin in drug formulations [37].

A literature survey of charge transfer complexation reactions of polyhalo/polycyanoquinones with basic nitrogenous centres reveals that 2,3-dichloro 5,6-dicyano 1,4-benzoquionone (DDQ) is one of the sensitive reagents among them which acts as an electron acceptor and yields more sensitive results in comparison to other polyhaloquinones [38–41]. In 1964, Jaroslav Bartos introduced ascorbic acid as a sensitive and economical reagent for the detection and determination of primary amino group in *N*,*N*-dimethyformamide medium [42]. Since then the mechanism of this reaction has not been exactly elucidated yet, so not much attention has been paid to the use of ascorbic acid as an economical spectrophotometric reagent for the



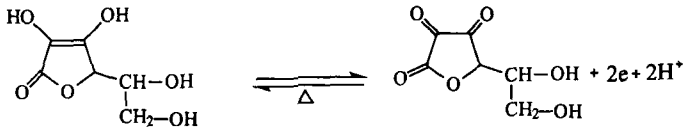
Scheme 1.

delete

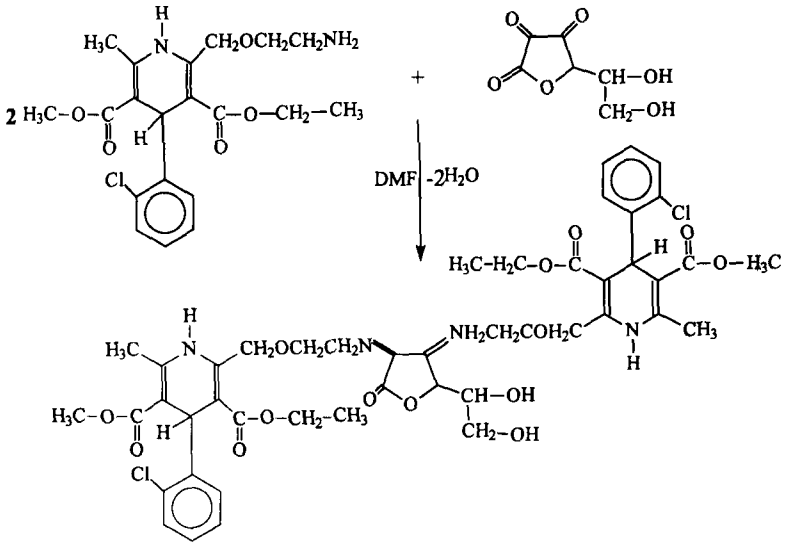
determination of reaction with amino group specially in pharmaceutical analysis.

This paper describes two sensitive, fast, simple and economical methods for the determination of amlodipine besylate in pure and dosage forms. The first method is based on the charge transfer complexation reaction of amlodipine with DDQ. The second procedure utilizes the reaction of primary $-NH_2$ group of the drug with ascorbic acid in *N*,*N*-dimethylformamide medium. The proposed methods are validated statistically.

I - Formation of dehydroascorbate from ascorbic acid (dehydrogenation)



II- Imine formation by coupling of amlodipine with dehydroascorbate



Scheme 2.

2. Experimental

2.1. Reagents and materials

All chemicals used were of AR-grade. Water was doubly distilled. A 0.1% solution of amlodipine besylate (Wockhardt Ltd., India) was prepared in chloroform. 0.1% solution of amlodipine besylate was also prepared in *N*,*N*-dimethylformamide (S.D. Fine Chem. Ltd., India). As reagents 0.05% DDQ (Fluka, Switzerland) solution in acetonitrile and 0.5 M aqueous sodium carbonate (E. Merck, India) solution were prepared for DDQ method. For the second method, 0.2% ascorbic acid was prepared by dissolving 100 mg of ascorbic acid (S.D. Fine) in 0.5 ml of water in a 50 ml standard flask and diluting to the volume with DMF.

2.2. Recommended procedure

2.2.1. DDQ method

2.2.1.1. Preparation of amlodipine base solution. In a 150 ml separatory funnel, 50 ml of 0.1% amlodipine besylate solution in chloroform was transferred followed by 75 ml of 0.5 M aqueous sodium carbonate solution. The content was mixed well and shaken for a few minutes. The organic layer was separated and dried over anhydrous sodium sulphate. A 25 ml portion of organic layer containing amlodipine base was evaporated to dryness and the residue was taken up with

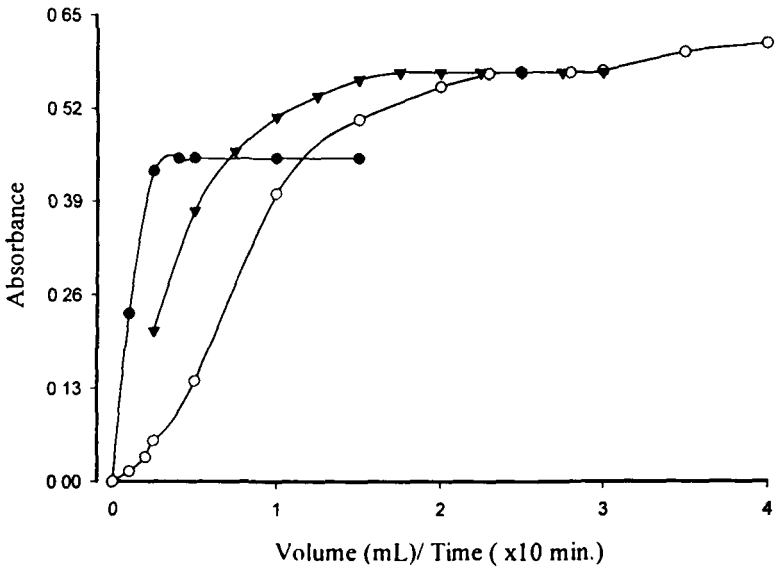


Fig 2. Effect of the volume of 0 05% DDQ (●) and 0 2% ascorbic acid (▼) and the heating time (○).

acetonitrile and transferred to 50 ml standard volumetric flask, diluting to volume. This corresponds to 0.05% amlodipine base solution.

2.2.1.2. Procedure for the determination. Aliquots of 0.05% amlodipine base corresponding to 1–125 μg ml^{-1} were transferred into a series of 5 ml volumetric flasks. 1.0 ml of 0.05% DDQ solution was added in each flask and diluted to volume with acetonitrile. The coloured product formed immediately and remained stable from 3 to 25 min. Therefore, the absorbances were measured within the stability period at 580 nm against the reagent blank prepared simultaneously.

2.2.2. Ascorbic acid method

Into a series of boiling tubes, aliquots of 0.1% amlodipine besylate solution in DMF (10–140 μg ml^{-1}) were pipetted. To each tube, 2.5 ml of 0.2% ascorbic acid solution was added. The total volume in each tube was maintained to 5 ml by adding DMF. The contents were mixed well and placed on a water bath maintained at 100±1 °C for 25 min. The solutions were cooled to room temperature. The reaction mixture and their corresponding washings were transferred and collected in a series of 10 ml volumetric flasks. They were diluted to volume with DMF. The absorbances were measured within the stability period of 4 h at 530 nm against the reagent blank treated similarly.

2.2.2.1. Analysis of pharmaceutical formulations. Ten tablets (claiming for 10 mg of amlodipine besylate per tablet) were finely powdered and extracted into sufficient volume of chloroform with shaking. The residue was filtered on whatmann filter paper no. 42 and the filtrate was diluted to 100 ml with chloroform. The solution of amlodipine base was prepared as discussed above and the drug was analyzed following the recommended procedure using DDQ as the reagent.

An accurately weighed portion of powdered tablets equivalent to 100 mg of amlodipine besylate was stirred with sufficient volume of DMF and left standing for 10 min. The residue was filtered on whatmann filter paper no. 42 and washed well with DMF. The filtrate and washings were diluted to volume in a 100 ml volumetric flask. The assay was completed following the recommended procedure using ascorbic acid as reagent.

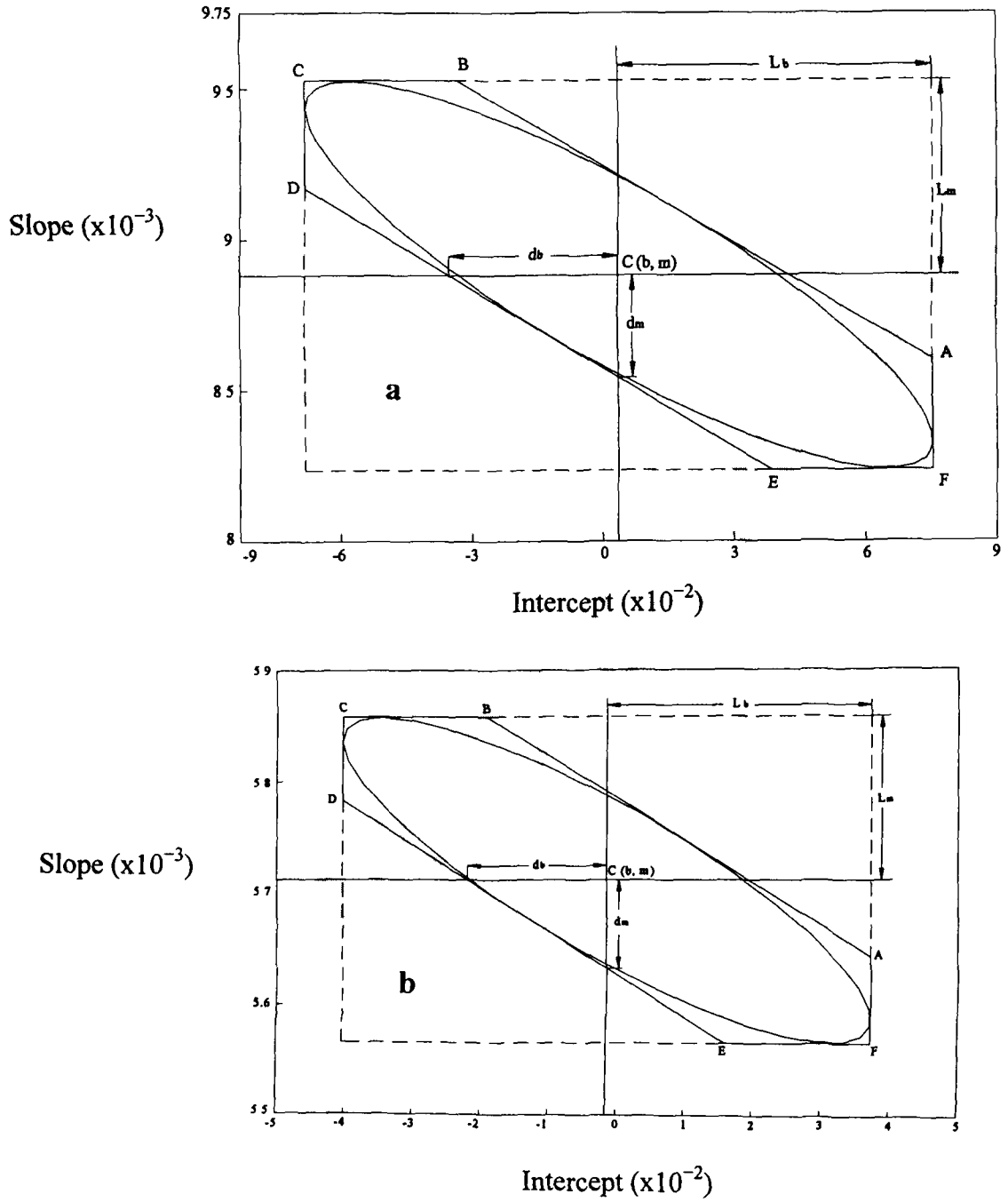


Fig. 3. Joint confidence region ($P = 0.05$) for the slope and intercept of regression line of DDQ method (a). Joint confidence region ($P = 0.05$) for the slope and intercept of regression line of ascorbic acid method (b).

3. Results and discussion

3.1. Reaction mechanism and IR studies

The molecular interactions between electron donors and acceptors are generally associated with the formation of intensely coloured charge transfer complexes or radical anions depending on the polarity of the solvent used. DDQ is a π-acceptor which readily forms charge transfer complexes with basic nitrogenous compounds as n-donors [38–41]. Some salts of amines do not react with σ- or π-acceptors because of non-availability of lone pair of electrons. In a similar manner, amlodipine besylate does not react with DDQ. In order to determine amlodipine besylate, the drug was dissolved in chloroform and shaken with a 0.5 M aqueous sodium carbonate solution. This treatment yielded amlodipine base in chloroform layer and evaporated to dryness. The residue was taken up in acetonitrile, a more polar solvent. The amlodipine base acts as an n-donor to form reddish violet coloured charge transfer complex with DDQ showing absorption maxima at 435, 550 and 580 nm (Fig. 1a). These bands may be attributed to the formation of DDQ radical anions, which probably resulted through the dissociation of the donor–acceptor complex in a highly polar solvent like acetonitrile. In order to avoid the maximum interference from the reagent blank, the absorption band at λ_{max} 580 nm was chosen for analytical studies. The Job's plot (Fig. 1b) suggested a donor to acceptor ratio of 1:2, confirming the presence of two n-donating centres in the amlodipine base molecule [43]. On the basis of the literature background and our experimental observations, a reaction mechanism is proposed and given in Scheme 1.

A purple red coloured product is obtained on heating amlodipine besylate with ascorbic acid in DMF, which absorbed maximally at 530 nm (Fig. 1c). On heating in a water-bath, the oxidation of ascorbic acid occurs mainly due to the formation of dehydroascorbic acid [44]. The carbonyl group further reacts with $-NH_2$ group of amlodipine to form a purple red coloured imine. The IR spectrum of amlodipine besylate displayed a band in the region 3120–2950 cm^{-1} attributed to N^+–H stretching mode. A sharp band at 1697 cm^{-1} may be due to C=O stretching vibration. The aromatic nature of the drug is characterized by the bands appearing in the region 1610–1450 cm^{-1}.The band at 1303 cm^{-1} and other bands in the region 1200–1000 cm^{-1} may be assigned to C–N (ring) and C–N (aliphatic amine) stretching vibrations [45]. The IR spectrum of the reaction product exhibits a broad band in the region 3600–3300 cm^{-1} which may be attributed to –OH and –CH stretching vibrations whereas N^+–H stretching mode disappeared. Another sharp and strong band at 1670 cm^{-1} suggested the formation of C=N group. The C=O stretching vibration is also shifted to a lower value and may appear in the C=N group region. The IR spectrum of the product has also indicated the OH deformation vibrations and the presence of C–O–C stretching appearing at 1256 and 1102 cm^{-1}, respectively. Thus the comparative study of the IR spectra of amlodipine besylate and the reaction product suggested an imine formation. The reaction mechanism is proposed in Scheme 2.

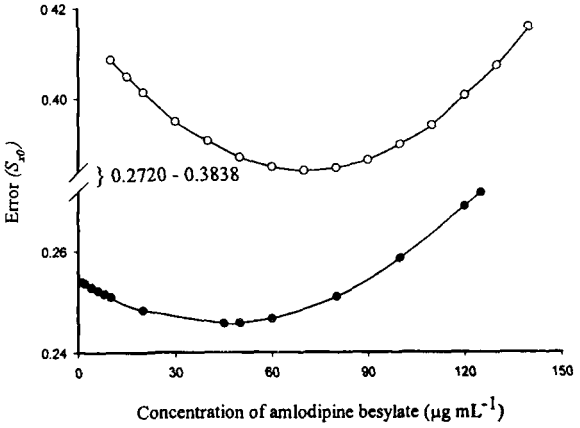


Fig. 4. Plot of error in the determination of the concentration of amlodipine besylate by DDQ method (●) and ascorbic acid method (○).

3.2. Optimization of variables

In DDQ method, the only effective variable is the concentration of DDQ since the reaction gets stabilized within 3 min and remains unaffected for a further 20 min. To study the effect of the

Table 1
Evaluation of the accuracy and precision of the two proposed procedures

Proposed methods	Amount taken (mg ml^{-1})	Amount found (mg ml^{-1})±S.D.[a]	RSD (%)[a]	SAE[b]	Confidence limit[c]
DDQ	10	10.01±0.06	0.64	0.03	0.061
	60	60.00±0.31	0.51	0.14	0.297
	100	100.03±0.54	0.54	0.24	0.516
Ascorbic acid	20	20.00±0.23	1.14	0.10	0.216
	80	80.03±0.46	0.57	0.20	0.434
	100	100.05±0.51	0.51	0.23	0.490

[a] Mean±S.D. for five determinations.
[b] SAE, standard analytical error.
[c] Confidence limit at 95% confidence level and four degrees of freedom ($t = 2.132$) [51].

concentration of DDQ, varying volumes of 0.05% reagent was mixed with 0.5 ml of drug in a 5 ml standard flask and diluted to volume with acetonitrile. The absorbance was measured after 3 min of mixing at 580 nm against the reagent blank. It was found that 0.5 ml of the reagent gave the highest absorbance (Fig. 2); above this volume the absorbance remains constant. A volume of 1.0 ml was, therefore, used in all further measurements.

To optimize heating time for ascorbic acid method, 1.0 ml of 0.1% amlodipine besylate was mixed with 2.5 ml of 0.2% ascorbic acid and heated at 100±1 °C. The absorbance was measured at 530 nm against the reagent blank as a function of heating time. The results (Fig. 2) show that the absorbance remains constant between 22 and 32 min of heating. There is an abrupt change in the absorbance above 32 min of heating and therefore, 25 min of heating time was used throughout the experiment.

In order to study the effect of volume of reagent on the absorbance, varying volume of 0.2% ascorbic acid was mixed with 1.0 ml of 0.1% amlodipine besylate in different boiling tubes and the contents were heated on the water bath at 100 ±1 °C for 25 min. The highest absorbance was obtained with 1.75 ml of the reagent (Fig. 2); above which the absorbance remains unaffected. 2.5 ml of the reagent was taken as optimum value.

3.3. Analytical data

Under the optimum experimental conditions, linear calibration graphs were obtained over the concentration ranges 1–125 and 10–140 μg ml^{-1} of amlodipine besylate with molar absorptivities of 0.60×10^4 and 0.32×10^4 l mol^{-1} cm^{-1} using DDQ and ascorbic acid, respectively. The calibration data were fitted by least square treatment and the regression equations obtained for DDQ and ascorbic acid methods were $A = 3.54 \times 10^{-3} + 8.88 \times 10^{-3}C$ and $A = -1.55 \times 10^{-3} + 5.71 \times 10^{-3}C$, respectively. In each case, the correlation coefficient was found to be 0.9999, indicating the good linearity of both the calibration graphs and the intercepts are all close to zero. The confidence intervals of intercepts at 95% confidence level were calculated (1.45×10^{-3} and 1.90×10^{-3} for DDQ and ascorbic acid methods, respectively) which confirmed that these are not different from zero. Thus the present methods are free from constant errors independent of the concentration of amlodipine besylate. There is also strong correlation existing between the slope and intercept. In order to judge the reliability of strong correlation of these parameters, more rigorous treatment of calibration data was made to draw a joint confidence region (Fig. 3a and b) following the method of Mandel and Linning [46]. The joint confidence region for slope and intercept is a tiltless ellipse having the point of best fit as its centre. It is evident from Fig. 3a and b that the points for which intercept is zero fall well within the ellipse.

The variance was calculated using the equation $S_o^2 = \Sigma(A_{exp} - A_{calc.})^2/n-2$ [47] and found to be 4.44×10^{-6} and 4.50×10^{-5} for DDQ and ascorbic acid methods, respectively. The small values of variance obtained for both the methods indicated

Table 2
Standard addition method for the determination of amlodipine besylate in dosage forms

Formulation name	DDQ method						Ascorbic acid method					
	Amount (μg ml^{-1})			Recovery (%) ± RSD (%)[a]	SAE[b]	Confidence limit[c]	Amount (μg ml^{-1})			Recovery (%) ± RSD (%)[a]	SAE[b]	Confidence limit[c]
	Taken	Added	Found ± S D[a]				Taken	Added	Found ± S D[a]			
Amdepin-10	25	25	50 05 ± 0 26	100 10 ± 0 53	0 12	0 251	15	15	30 03 ± 0 29	100 17 ± 0 98	0 13	0 280
	35	35	70 01 ± 0 32	100 01 ± 0 45	0 14	0 303	45	45	89 83 ± 0 49	99 81 ± 0 55	0 22	0 468
Amlogard-10	25	25	50 06 ± 0 25	100 12 ± 0 49	0 11	0 234	15	15	30 10 ± 0 29	100 33 ± 0 95	0 13	0 274
	35	35	70 01 ± 0 38	100 03 ± 0 54	0 17	0 359	45	45	90 08 ± 0 51	100 09 ± 0 56	0 23	0 486
Amlong-10	25	25	49 75 ± 0 30	99 50 ± 0 60	0 13	0 284	15	15	29 96 ± 0 31	99 87 ± 1 05	0 14	0 299
	35	35	69 54 ± 0 34	99 34 ± 0 49	0 15	0 328	45	45	89 86 ± 0 47	99 84 ± 0 52	0 21	0 446
Amlopin-10	25	25	50 03 ± 0 29	100 06 ± 0 59	0 13	0 281	15	15	30 00 ± 0 31	100 01 ± 1 02	0 14	0 293
	35	35	69 85 ± 0 36	99 79 ± 0 52	0 16	0 346	45	45	89 97 ± 0 45	99 97 ± 0 49	0 20	0 424
Amlopres-10	25	25	49 93 ± 0 31	99 86 ± 0 62	0 14	0 294	15	15	29 98 ± 0 27	99 93 ± 0 91	0 12	0 260
	35	35	69 97 ± 0 37	99 96 ± 1 53	0 17	0 351	45	45	89 91 ± 0 48	99 90 ± 0 53	0 21	0 453
Myodura	25	25	50 08 ± 0 29	100 16 ± 0 58	0 13	0 275	15	15	30 01 ± 0 28	100 03 ± 0 95	0 13	0 271
	35	35	70 14 ± 0 33	100 20 ± 0 46	0 15	0 312	45	45	90 05 ± 0 44	100 05 ± 0 49	0 20	0 419

[a] Mean ± S D for five determinations
[b] SAE, standard analytical error
[c] Confidence limit at 95% confidence level and four degrees of freedom ($t = 2\ 132$) [51]

Table 3
Comparison of the two proposed methods with the reference method [30]

Pharmaceutical preparations	Labelled amount (mg)	DDQ method				Ascorbic acid method				Reference method	
		Recovery (%)[a]	RSD (%)[a]	t-value[b]	F-value[b]	Recovery (%)[a]	RSD (%)[a]	t-value[b]	F-value[b]	Recovery (%)[a]	RSD (%)[a]
Amdepin	10	100.13	0.73	0.0404	4.51	99.90	0.59	0.7370	2.93	100.11	0.34
Amlogard	10	100.20	0.67	0.2952	4.20	99.98	0.57	0.3664	2.98	100.09	0.33
Amlong	10	99.95	0.32	0.0614	1.85	99.98	0.36	0.0351	2.41	99.98	0.24
Amlopin	10	99.96	0.38	0.0434	1.11	99.72	0.84	0.6133	4.46	99.97	0.40
Amlopres	10	99.98	0.51	0.5369	1.22	99.75	0.95	0.8667	2.81	100.14	0.55
Myodura	10	100.19	0.68	0.4451	2.72	99.80	0.67	0.6463	2.60	100.03	0.41

[a] Average of five independent analyses.
[b] Theoretical t-value (DOF = 8) and F-value (DOF = 4,4) at 95% confidence level are 1.860 and 6.39, respectively [51].

negligible scattering of the experimental data points from the line of best fit. The values of correlation coefficients were not sufficient enough to evaluate the linearity of the calibration graphs. The linearity was evaluated by the percent relative standard deviation of the slope (Sb_{rel}%) [48]. The values were found to be 0.11 and 0.18 for DDQ and ascorbic acid methods, respectively, which indicated better linearity of the former method.

The statistical analysis of the calibration data also allows the calculation of error (S_{x_0}) in the determination of a given concentration of amlodipine besylate and may be helpful to establish the confidence limits at the selected levels of confidence in the determination of unknown concentrations. These parameters were evaluated by using the following formula [49].

$$S_{x_0} = \frac{S_{y/x}}{b}\left[1 + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}\right]^{1/2} \quad (1)$$

where $\bar{y}$ and $\bar{x}$ are the average absorbance and concentration values, respectively for n standard specimens. Fig. 4 shows the graphs of S_{x_0} versus concentration of amlodipine besylate. The error is minimum when the actual absorbance is equal to the average absorbance, which corresponds to about 45 and 70 μg ml^{-1} for DDQ and ascorbic acid methods, respectively. The accuracy and precision of the proposed methods were evaluated by the repeated analyses at three different concentration levels. The results are summarized in Table 1. The standard deviations, relative standard deviations and standard analytical errors [50] can be considered to be very satisfactory.

As an additional demonstration of accuracy, recovery experiments were performed by adding a known amount of amlodipine besylate to the preanalyzed dosage forms. The results showed (Table 2) that the mean recoveries were in the range of 99.34–100.20%. No interference from the common excipients was observed.

The methods were successfully applied to the determination of amlodipine besylate in pharmaceutical formulations. The results of the proposed method (DDQ or ascorbic acid) were compared with those of the reference method [30]. Table 3

shows that the calculated t- and F-values are less than the theoretical ones, confirming accuracy and precision at 95% confidence level

Under the experimental conditions described, the linearity and sensitivity were the best with the charge transfer complex formation procedure Both the proposed spectrophotometric methods are simple, sensitive and reproducible Moreover, these procedures are likely to be very suitable for the routine analysis of amlodipine besylate in dosage forms

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